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TABACHNICK, IRVING I. A., R. E. PARKER, J. WAGNER AND PAUL Z. ANTHONY
A Re-Evaluation of Calorigenic Action of L- and D-Isomers of Thyroxine.. 155
McDONALD, D. F. AND M. J. LATTA
Anerobic Glycolysis of Human Prostatic Adenoma: *In Vitro* Inhibition by Estrogen and by Androgen and Estrogen 159
VELARDO, JOSEPH T., FREDERICK L. HISAW AND ARLEY T. BEVER
Inhibitory Action of Desoxycorticosterone Acetate, Cortisone Acetate, and Testosterone on Uterine Growth Induced by Estradiol-17β..... 165
ULRICH, FRANK AND C. N. H. LONG
Effects of Stress on Serum C^{14} Levels in Rats Following Administration of Hydrocortisone-4-C^{14} and Corticosterone-4-C^{14}..... 170
ELLIOTT, JOSEPH R. AND SMITH FREEMAN
Parathyroid Function and the Plasma Citric Acid and Calcium Response .. 181
FREEMAN, SMITH AND JOSEPH R. ELLIOTT
The Effect of Fluoroacetate upon the Plasma Citrate Response to Parathyroidectomy and Nephrectomy..... 190
ELLIOTT, JOSEPH R. AND SMITH FREEMAN
Relative Effect of Vitamin D and Parathyroid Extract on Plasma Calcium and Citric Acid of Normal and Thyroparathyroidectomized Dogs..... 196
SKELTON, FLOYD R. AND JAMES GUILLEBEAU
The Influence of Age on Development of Adrenal-Regeneration Hypertension 201
MARUYAMA, KOSÇAK AND HIDESHI KOBAYASHI
Effect of Thyroxine on Latent Apyrase Activity of Canary Liver Mitochondria 213
SWANSON, HEIDI EARTLY
Interrelations Between Thyroxin and Adrenalin in the Regulation of Oxygen Consumption in the Albino Rat..... 217
SWINGLE, W. W., L. J. BRANNICK, A. F. PARLOW, C. BAKER AND STEPHEN LEBRIE
Life Maintenance and Mineralocorticoid Activity of Some New Derivatives of Cortisone, Hydrocortisone and DOC..... 226
SEGALOFF, ALBERT, SANFORD L. STEELMAN AND ALBERT FLORES
Prolactin as a Factor in the Ventral Prostate Assay for Luteinizing Hormone 233
ADROUNY, GEORGE A. AND JANE A. RUSSELL
Growth Hormone and Nutritional Status on Cardiac Glycogen in the Rat. 241
NOTES AND COMMENTS
ALBRIGHT, EDWIN C., F. C. LARSON, KENKICHI TOMITA AND HENRY A. LARDY
Enzymatic Conversion of Thyroxine and Triiodothyronine to the Corresponding Acetic Acid Analogues..... 252
BAKER, BURTON L., J. H. KLEGMAN, R. G. LOGAN AND R. L. GARNER
Reduction in Parotid Amylase after Hypophysectomy..... 254
STEELMAN, SANFORD L., THOMAS L. KELLY, ALBERT SEGALOFF AND G. F. WEBER
Isolation of an Apparently Homogeneous Follicle Stimulating Hormone 256
VULPÉ, M., D. USHER AND C. P. LEBLOND
Influence of the Extirpation of Male Accessories on the Occurrence of a "Soft Calculus" in the Urinary Bladder of the Rat..... 257
INGLE, DWIGHT J.
Urinary Glucose and Tumor Growth in Diabetic Rats..... 259
SULKOWITCH, HIRSH
The Colorimetric Estimation of the Epinephrines, Dihydroxyindoles and Their Quinones with Aromatic Orthodiamines..... 260
THE ENDOCRINE SOCIETY, 1957 ANNUAL MEETING..... 263
UROLOGY AWARD..... 271
COLLEGE OF AMERICAN PATHOLOGISTS..... 271
AMERICAN PSYCHOSOMATIC SOCIETY..... 272

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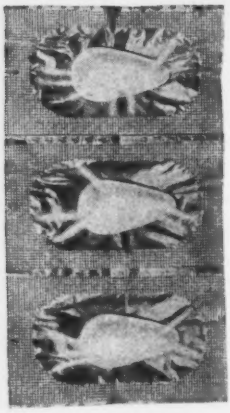

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A RE-EVALUATION OF THE CALORIGENIC ACTION OF THE L- AND D-ISOMERS OF THYROXINE

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THERE has been a generalized lack of agreement among investigators in different laboratories regarding the activity of D-thyroxine as compared to L-thyroxine (1–10; cf. Table 1). Since most of the estimates of potency were obtained without the use of precise statistical assay methods, these divergent results are understandable.

TABLE 1. A COMPARISON OF THE EFFECTS OF D-, DL-, AND L-ISOMERS OF THYROXINE REPORTED BY PREVIOUS INVESTIGATORS

Investigators	Physical data				Biologic data			
	Isomers compared	Light source	Specific rotation		Test unit	Protocol	Potency ratio	
			D-	L-			L/D	L/DL
Gaddum (1929)	D-vs L-	Mercury	+3.0°	−3.1°	Rat Tadpole	Single, SC Dose In bath	1.5–3.0 1.2–2.0	— —
Salter *et al.* (1935)	D-vs L-	—	—	—	Human myxedema	I. V. q.d.	1.0	—
Foster *et al.* (1936)	DL-vs L-	Sodium		−4.4°	Guinea pig	SC, q.d. for 14 days	—	2.0
Reinecke and Turner (1943)	DL-vs L-	Sodium		−4.2°	Guinea pig	SC, q.d. for 12 days	—	2.0
Reinecke and Turner (1945)	DL-vs L-	Sodium		−4.2°	Thiouracil-treated chickens Tadpole	SC, q.d. for 14 days In bath	— —	2.0 2.0
Griesbach *et al.* (1947)	DL-vs L-	—	—	—	Rat pituitary basophils	Methyl thiouracil, SC, q.d. for 17–20 days	—	1.5
Smith (1947)	D-vs L-	—	—	—	Mouse anoxia	—	D is inactive	—
Pitt-Rivers and Lerman (1948)	D-vs L-	Sodium	+5.5°	—	Human myxedema	I. V. q.d.	8–10	—
Means (1948, p. 63)	D-vs L-	—	—	—	Human myxedema	—	6	—
Griesbach *et al.* (1949)	D-vs L-	—	—	—	Rat pituitary basophils	Methyl thiouracil, SC, q.d. for 17–20 days	3	—

Received November 25, 1955.

[1] Present address: Schering Corporation, Bloomfield, New Jersey

We have therefore thought it important to reevaluate the calorigenic action of highly purified samples of L- and D-thyroxine,[2,3] by means of a modified mouse anoxia technique and by a statistically valid slope-ratio assay to determine the potency ratio.

METHODS AND MATERIALS

Animals and Materials. Male albino mice of a Carworth strain weighing 18–24 gm. were used throughout this study. The mice were housed in an air-conditioned room in our laboratory for several days before use. Purina chow and tap water were fed *ad libitum*.

Thyroxine solutions were prepared by dispersing the crystalline hormone in small amounts of 0.9% saline and adding 1N sodium hydroxide until complete solubility was achieved. The pH was then adjusted to 8.0 with 1N hydrochloric acid and 0.9% saline added to obtain the desired volume. All injections were administered subcutaneously in volume of 0.2 ml.

Measurement of Calorigenic Action. The mouse anoxia method of Burn, Finney and Goodwin (11) was used throughout this study. This procedure measures the survival time of individual mice in sealed mason jars. Survival time is defined as the length of time elapsing from the moment the jar is sealed to the cessation of respiration. The term, calorigenic action, will be used interchangeably with survival time since all evidence indicates that the latter is a function of the calorigenic action of thyroid hormone.

Five groups were used to determine relative potency: (a) Saline controls, (b) 20 μg. Na L-thyroxine per mouse, (c) 40 μg. Na L-thyroxine per mouse, (d) 60 μg. Na D-thyroxine per mouse and (e) 120 μg. Na D-thyroxine per mouse. These agents were administered every second day for a total of 3 doses. On the eighth day, the mice were assigned at random to mason jars and the jars sealed. The data obtained was analyzed by the slope-ratio technique described by Bliss (12) and by Finney (13).

Duration of the calorigenic effects of the two isomers was determined by administering single injections of either 250 μg. or 500 μg. per mouse of either isomer to groups of 20 mice 24, 48, 72 and 96 hours before they were placed in jars. Survival times were ascertained as described above.

Specific Rotation of D-Thyroxine. The pH of the D-thyroxine solution remaining after each experiment was adjusted to 4.0 with 3N acetic acid. The white precipitate obtained was collected by filtration and washed with water. After drying, the solid was dissolved in an acidic solvent consisting of one part 2N nitric acid, three parts 95% ethyl alcohol and two parts of ethyl acetate. Readings were made in a 1 dm. tube.

RESULTS

Potency by Mouse Anoxia. Figure 1 shows that the slope of the D-isomer dose-response curve was less steep when compared to the slope of the dose-response curve of the L-isomer. In addition, the two lines intersect at zero-dose (control). These attributes fulfill the criteria essential to a slope-ratio analysis and consequently this method of analysis was performed (Table 2). Since neither the blanks nor the intersection components were signifi-

[2] Unpublished work of Paul Z. Anthony, L. J. Sacco, Jr., and L. G. Ginger, Baxter Laboratories, Morton Grove, Ill.

[3] The L-thyroxine used was the active ingredient of Synthroid®, a tableted formulation of Na L-Thyroxine pentahydrate, available from Travenol Laboratories, Inc., a subsidiary of Baxter Laboratories, Inc.

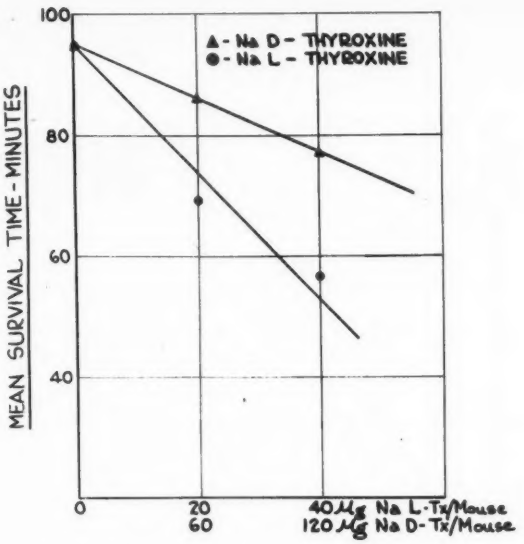

FIG. 1. Dose-response curves for Na L- and D-thyroxine. Each point represents the mean survival of a group of 20 mice.

cantly greater than the error component, and the regression was significant, the data could be used to estimate relative potency as follows:

Regression coefficients for the L- and D-isomers (b_L and b_D respectively) were calculated by the method of Finney (13) as follows:

$$b_L = -40.954$$

$$b_D = -16.764$$

$$J' = \text{relative potency (coded units)} = b_L/b_D = 2.44$$

$$J = \text{relative potency (original units)} = J' \times \frac{I_D}{I_L} = 2.44 \times 3 = 7.32$$

where

$$\frac{I_D}{I_L} = \text{the ratio of the doses of the two isomers} = \frac{60}{20} \text{ or } \frac{120}{40}.$$

TABLE 2. SLOPE RATIO ANALYSIS OF VARIANCE OF THE EFFECTS OF Na D- OR L-THYROXINE IN MICE

Term	D.F.	Sum of Squares	Mean squares	F
Regression	2	18,974.4	9,487.2	32.43*
Blanks	1	321.2	321.2	1.10
Intersection	1	378.7	378.7	1.29
Error	95	27,791.9	292.2	—
Total	99	47,466.2		

* $p < 0.001$.

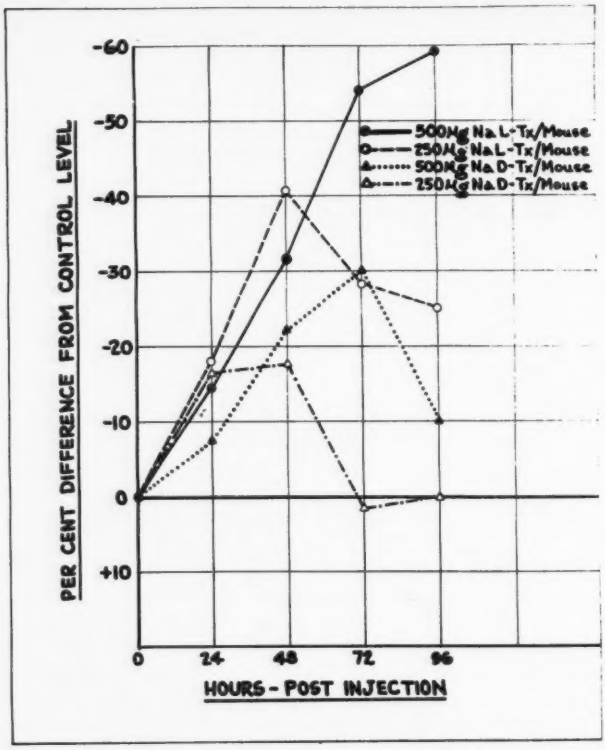

FIG. 2. The effect on survival time (per cent change from control) of a single injection of Na L- or D-thyroxine 24, 48, 72, or 96 hours before the anoxia was begun. Each point is derived from the mean survival time of a group of 17 to 20 mice.

Thus, the Na L-isomer was found to be 7.3 times more potent than the Na D-isomer, with 95% fiducial limits of 4.9 to 16.6 (14).

Duration of Calorigenic Action As shown in Figure 2, no distinction could be made between the response induced by single doses of either D- or L-thyroxine 24 hours after treatment. However, 48 hours after the administration of either of the two isomers, the survival time of mice treated with L-thyroxine was significantly less when compared to D-thyroxine. At the end of 96 hours the calorigenic action of L-thyroxine was still apparent whereas the D-isomer no longer produced a significant effect. Thus, there would appear to be a qualitative difference between the two isomers of thyroxine.

Optical Acitivity. As indicated in Table 3, racemization was not observed three weeks after preparation of an aqueous solution of Na D-thyroxine.

DISCUSSION

Gaddum (1929) observed that the activity of the D-isomer approached that of the L-isomer with progressively smaller doses of hormone. This ob-

TABLE 3. SPECIFIC ROTATION, $[\alpha]_D^{24}$, OF Na D- OR L-THYROXINE UNDER VARIOUS CONDITIONS

	pH of medium	$\left[\alpha\right]_D^{24}$	
		L-	D-
Laboratory average	Alkaline Acid	−5.1° to −5.7° +13.0° to +15.0°	+5.0° to 6.5° −13.0° to −15.0°
Experimental Run Initial Final	 Acid Acid		 −13.8° −14.8°

servation may be explained in part by the work of Rall (15), who demonstrated D-thyroxine has approximately one-fourth the serum half-life of L-thyroxine, and the results of this study (cf. Fig. 1). The estimate of relative potency of the two isomers reported in this communication would appear to be similar to that obtained by Pitt-Rivers and Lerman (8).

The calorigenic action of D-thyroxine could be attributed to a conversion to the L-form. Our studies suggest that *in vitro* racemization did not occur. However, *in vivo* racemization is still possible, although Pitt-Rivers and Lerman (8) considered this unlikely. If racemization did occur *in vivo*, one would expect the response of the two isomers to parallel one another. However, as seen in Figure 2, the measured responses of the two isomers upon single injection were similar only after 24 hours. At 48, 72, and 96 hours, a large spread between the response to the isomers was observed.

Martius and Hess (16) have shown that thyroxine can inhibit oxidative phosphorylation *in vitro*, an effect which may be related to the main physiological action of the thyroid hormone. Klemperer (17) reported that D-thyroxine, *in vitro* was not as potent an uncoupling agent as is the L-isomer. However, Lardy and Maley (18, 19) found the two isomers to be of equal potency in dissociating oxidation from phosphorylation.

Lastly, our data indicate that the mouse anoxia assay is sufficiently sensitive to measure the activity of the D-isomer, contrary to the report of Smith (7).

SUMMARY

1. Using the mouse anoxia assay, Na L-thyroxine was found to be seven times more potent than Na D-thyroxine.

2. Differences in the nature of the response elicited by the two isomers were demonstrated. Upon single injection, the calorigenic action of L-thyroxine was more prolonged than the action of the D-isomer. Upon repeated injection, the slope of the dose-response curve of the D-form was less steep than the slope of the L-form.

ADDENDUM

P. Starr and R. Leibhold-Schueck have reported recently (Thirty-Seventh Meeting, the Endocrine Society, Atlantic City, N. J.) that the

L-isomer is about 5 times as potent as the D-isomer. They observed that the D-isomer administered orally produced a protein bound iodine concentration only one-third that achieved with the same amount of the L-form. This phenomenon may be associated with the shorter serum half-life of the D-isomer as demonstrated by Rall (15).

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ANEROBIC GLYCOLYSIS OF HUMAN PROSTATIC ADENOMA: *IN VITRO* INHIBITION BY ESTROGEN AND BY ANDROGEN AND ESTROGEN[1]

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THE clinical usefulness of estrogens in treatment of carcinoma of the prostate scarcely needs documentation, but the mechanisms whereby the effects are produced are incompletely understood. Indirectly, pituitary inhibition by estrogens may decrease gonadotrophin production which results in reduced testosterone production by the testis. A direct estrogen-androgen antagonism is postulated by Huggins (8), for Huggins and Clark (6) have shown that estrogen can nullify the stimulatory effect of testosterone on the dog prostate "*in vivo*". In the castrate human with active prostatic carcinoma, administration of estrogens further inhibits the prostatic carcinoma. This may be a direct effect on the carcinoma, or it may be due to suppression of the pituitary lactogenic hormone, which Grayhack, Kearns, Bunce and Scott (4) have experimentally shown may enhance prostatic growth. Flocks, Marberger, Begley and Prendergast (3) have found that high doses of diethylstilbestrol may inhibit prostatic carcinoma even in the absence of testes and adrenals. The tissue culture experiments of Lasnitzki (10) indicate that estrogen may act directly on the mouse prostate. Lehninger and Scott (11) did not observe inhibition of the respiration of adenomatous human prostatic tissues when α-estradiol up to $7.4\times10^{-5}M$ was used. Barron and Huggins (1) found that the prostates of castrate and diethylstilbestrol-treated dogs showed increased aerobic carbohydrate metabolism, but anerobic glycolysis remained unchanged. Histological regression of the epithelium of human adenomatous prostates has been observed to follow the systemic administration of estrogen (7).

It might be inferred from the evidence at hand that the estrogens must have a direct local action on the prostate, normal, benign or malignant. This has not been definitely established. The present study experimentally proves these inferences and suggests the order of magnitude of estrogen levels required to consistently induce inhibition of the anerobic carbohydrate metabolism of the human adenomatous prostate.

Received December 14, 1955.

[1] This investigation was supported in part by grants-in-aid from the National Institutes of Health, U. S. Public Health Service, the John and Mary R. Markle Foundation and by funds from the American Cancer Society, as recommended by the Committee on Growth.

METHODS

Methods were similar to those previously described (12). The diethylstilbestrol[2] and testosterone[3] were ground well in a mortar and pestle and suspended in Krebs-Ringer bicarbonate buffer. Natural estrogens (Premarin)[4] were soluble in buffer and were diluted directly to the desired concentration. Where testosterone and either natural estrogen or diethylstilbestrol were added simultaneously, each was made up in its own solution or suspension with Tween 80[5] (for testosterone and diethylstilbestrol only), and 0.2 ml. of the appropriate solution was added to each of 2 side-arms on the reaction vessels. The test compounds were always tipped in simultaneously at the beginning of the timed metabolism period. The millimolar concentration of natural estrogen was expressed as millimoles of estrone equivalent.[6] Premarin was chosen as a source of natural estrogen because of its stability and solubility. The percent inhibition was calculated by dividing the average decrease in $Q_{CO_2}^{N_2}$ of the experimentals below the average control value by the average control value. All levels were run in triplicate or quadruplicate.

RESULTS

Diethylstilbestrol

Diethylstilbestrol inhibited anerobic glycolysis of human prostatic adenoma slices at levels above 2 μg./ml. At 0.2 μg./ml. significant ($P < .02$)

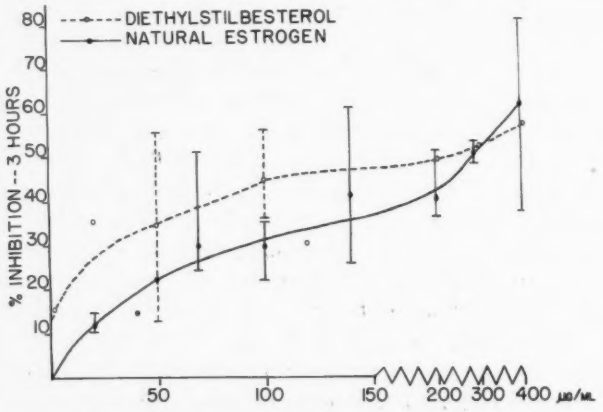

FIG. 1. Percent inhibition of human prostatic anerobic glycolysis by varying final concentrations of natural (equine) and synthetic estrogen.

stimulation was observed during the second and third hours in one experiment. There was some proportionality of response at levels between 50 and 400 μg./ml. (Fig. 1). The degree of inhibition was 12 to 57% of control values.

Two different specimens of normal human prostate in the presence

[2] Lot No. P-20999, E. R. Squibb and Sons, New York.

[3] Eli Lilly and Company, Indianapolis, Indiana.

[4] Ayerst, McKenna and Harrison, Ltd., New York.

[5] Tween 80 did not inhibit anerobic glycolysis in the dilute quantities used (2).

[6] The potency of conjugated equine estrogens (Premarin) is expressed in miligram of sodium estrone sulfate biological equivalents.

of diethylstilbestrol showed inhibitory effects of the same order of magnitude as that observed on adenomatous prostatic slices. Twenty μg. to 200 μg./ml. produced 42 to 47% inhibition in 3 hours.

The inhibitory effect of diethylstilbestrol on $Q_{CO_2}^{N_2}$ of adenomatous prostatic tissue was reduced by washing the tissue and replacing it in fresh buffered substrate. After two hours exposure to 100 μg. diethylstilbestrol/ml., prostate slices showed 34% inhibition. After washing and replacing in new buffer without diethylstilbestrol, the inhibition was 11% of control slices similarly treated.

Natural Estrogen[6] *(Premarin)*

The inhibitory effect of the natural soluble estrogens (Premarin) was observed to show greater variability of results between 10 and 50 μg./ml. than those observed with diethylstilbestrol. At 20 μg., two of four trials resulted in significant stimulation ($P < .05$). One of two trials at 40 μg. resulted in significant stimulation of +37% in three hours ($P < .02$). Between 50 and 400 μg./ml., there was consistent progressively greater inhibition (Fig. 1). During the three hour observation period the greatest degree of inhibition was noted in the first hour. Of 27 tissue specimens observed for three hours, 17 showed continued cumulative increase in the percent inhibition over controls, six showed no change after one hour, and four showed slight recovery, as manifest by decreased cumulative inhibition between first and third hour.

When diethylstilbestrol and natural estrogen were compared on a millimolar basis in the middle range, they were found to be of similar inhibitory potency. However, the highest levels of inhibition observed were found with the natural estrogens.

Simultaneous Androgen-Estrogen Administration

When prostatic adenoma slices were simultaneously treated with testosterone and diethylstilbestrol, the observed inhibition of $Q_{CO_2}^{N_2}$ was never as great as the sum of the inhibition for each of the hormones separately (Table 1).

Natural estrogen and testosterone given together in equimolar quantities usually caused less inhibition than the sum of the inhibition of the two given separately. The combined effect of testosterone plus natural estrogen was greater than the individual effect of either testosterone or natural estrogen given separately. Ratios of testosterone to natural estrogen of 1:2, 4:1 and 12.5:1 gave results similar to the 1:1 ratio. When the ratio was 2:1 and 25:1 the effect of combined treatment more nearly approximated the results of testosterone alone.

DISCUSSION

The anerobic glycolysis of human prostatic adenoma is its most active carbohydrate metabolic process. Barron and Huggins (1) have reported

TABLE 1. COMPARISON OF AVERAGE INHIBITION OF $Q_{CO_2}^{N_2}$ OF HUMAN PROSTATIC ADENOMA SLICES BY TESTOSTERONE, NATURAL AND SYNTHETIC ESTROGEN AND BY COMBINATIONS OF TESTOSTERONE AND ESTROGEN

Concentration μg./ml. buffer or mM as indicated	Percent inhibition				
	Testosterone alone	Diethylstilbestrol alone	Natural estrogen alone	Both[1]	Theoretical sum[2]
1:1 Ratio					
50 μg. each	56	43		65	92
100 μg. each	67	46		67	114
200 μg. each	72	50		76	148
0.173 mM each	58		26	64	84
0.347 mM each	57		38	72	95
0.695 mM each	63		53	79	116
1:2 Ratio					
0.173 mM T[3] & 0.347 mM NE[4]	53		42	61	95
0.695 mM T & 1.390 mM NE	63		70	77	133
100 μg. T & 200 μg. NE	66		42	77	107
1:4 Ratio					
100 μg. T & 400 μg. NE	87		81	72	168
50 μg. T & 200 μg. NE	27		36	53	63
2:1 Ratio					
0.347 mM T & 0.173 mM NE	72		45	78	117
0.695 mM T & 0.347 mM NE	67		35	69	102
4:1 Ratio					
0.695 mM T & 0.173 mM NE	69		45	88	114
1.390 mM T & 0.347 mM NE	59		35	66	93
25:1 Ratio					
100 μg. T & 4 μg. NE	40		0	40	40
12.5:1 Ratio					
50 μg. T & 4 μg. NE	32		0	44	32

[1] The actual inhibition when the androgen and estrogen were tipped in simultaneously.
[2] The sum of inhibition produced by testosterone alone and estrogen alone.
[3] T = testosterone.
[4] NE = natural estrogen.

that, whereas respiration was 1.95 μl. per mg. per hour and aerobic glycolysis was 2.2, the anerobic glycolysis was 5.39 μl. per mg. per hour. We have reported similar control results (12). A high anerobic glycolysis is also a metabolic path characteristic of neoplasm. The interesting dual action of natural and synthetic estrogens on prostatic anerobic glycolysis, depending on concentration, has a biological parallel in the mouse prostate tissue cultures of Lasnitzki. She found that levels of 2 μg./ml. of estrone stimulated hyperplasia, whereas 4 μg./ml. caused atrophy and squamous metaplasia (10). The direct "*in vitro*" effectiveness of natural and synthetic estrogen on the prostate differs from their "*in vivo*" uterine stimulating properties, which Szego and Roberts (15) found did not occur in the absence of the liver.

To extrapolate from "*in vitro*" to "*in vivo*" may not be justified. If it were, the following might be true. Since natural estrogens are found by

Roberts and Szego (14) and Riegel and Mueller (13) to be lipoprotein-bound, it would seem that the dose to be given by parenteral administration should be calculated on the basis of the plasma volume. If 5% of the body weight be chosen as a representative figure for plasma volume, a 70 kg. human should have the administered dose distributed in 3500 ml. of plasma. On this basis, the amount of diethylstilbestrol required to give plasma levels of 50 μg./ml. to 200 μg./ml. would be 175 to 700 mg. respectively. Flocks, Marberger, Begley and Prendergast (3) gave 250 to 1250 mg. of diethylstilbestrol diphosphate intravenously to 63 patients who had carcinoma of the prostate. The blood levels achieved are well above those required to consistently inhibit anerobic glycolysis of human prostatic adenoma slices "*in vitro*". Hertz (5) reported consistent blood levels over 100 μg./ml. following intravenous infusions of 1 gm. of potassium estrone sulfate per day for 7 to 10 days.

Simultaneous "*in vitro*" androgen and estrogen administration usually resulted in increased inhibition of anerobic glycolysis. The evidence suggests that both may inhibit at the same point in the carbohydrate cycle, since the inhibition of both were usually only slightly greater than the highest single effect. It does not seem that androgen and estrogen are mutually antagonistic with respect to inhibition of prostatic anerobic glycolysis. Although Kaufman and Goodwin (9) have found mixtures of androgen and estrogen to have some merit in treatment of clinical benign prostatic hypertrophy, there is little to suggest that such might be the case from the present study. From our results, it would seem that testosterone alone is the most effective inhibitor. However, the dose required to obtain inhibitory levels would be of the order of 5 mg. per kg. body weight intravenously.

SUMMARY

1. High levels of natural or synthetic estrogens significantly inhibited the anerobic glycolysis of human prostatic adenoma slices "*in vitro.*"

2. Low concentrations of estrogen occasionally stimulated increased anerobic glycolysis.

3. Combinations of androgen and estrogen usually slightly increased the degree of inhibition achieved by androgen alone. No evidence of estrogen-androgen antagonism or synergism was found.

4. Estimation of clinical doses required to achieve the necessary inhibitory levels was attempted.

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INHIBITORY ACTION OF DESOXYCORTICOSTERONE ACETATE, CORTISONE ACETATE, AND TESTOSTERONE ON UTERINE GROWTH INDUCED BY ESTRADIOL-17β[1]

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IT HAS been found that the three natural estrogens, estradiol-17β, estrone, and estriol show certain interactions in which one may limit the effects of another in the promotion of uterine growth in castrated rats (3, 4, 5). It also has been reported that several nonestrogenic steroids decrease the growth response of the rat uterus when administered concurrently with estradiol and estrone (6, 7). A brief abstract containing some of the data used in this report on the inhibitory effects of desoxycorticosterone acetate (DCA), cortisone acetate and testosterone on the action of estradiol-17β has been published (7) while the present paper deals with these effects in more detail.

PROCEDURE

Virgin rats 100 days of age were ovariectomized and beginning one week later, 0.1 μg. estradiol-17β was injected subcutaneously daily for three days concurrently with graded doses of the compounds mentioned above. The steroids were dissolved in sesame oil, with the exception of cortisone acetate which was suspended in an aqueous vehicle.[4] Each preparation was injected at a separate site in doses of 0.1 cc., except cortisone acetate which was given in 0.2 cc., and the animals were killed 24 hours after the last injection. The wet and dry weights of the uteri were recorded. Nitrogen determinations were made but since these data so closely parallel those for uterine dry weights they have been omitted. Each point on the graphs of data represents the arithmetical mean of at least six animals while those for the castrated controls and those for estradiol alone represent 39 and 57 animals, respectively.

Results.—Dose-response curves for the three steroids, desoxycorticosterone acetate (DCA), cortisone acetate and testosterone, showed marked

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[4] The authors wish to thank Schering Corporation, Bloomfield, New Jersey for generously supplying testosterone, desoxycorticosterone acetate, and estradiol-17β and Merck and Company, Rahway, New Jersey, for cortisone acetate.

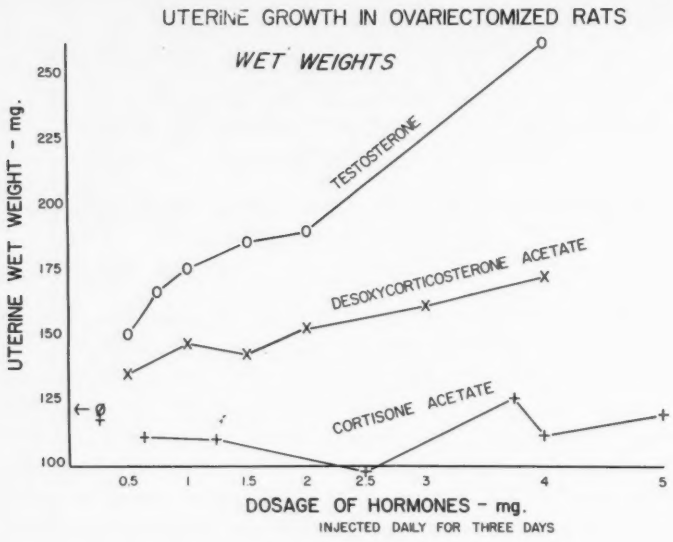

FIG. 1

differences in ability to promote uterine growth (Fig. 1). Of these, testosterone was the most effective. Doses of 0.5 mg. produced significant increases in the wet weight of the uterus (150 mg.) over that of castrated controls (122 mg.) and increments of 0.5 mg. caused progressively greater increases.

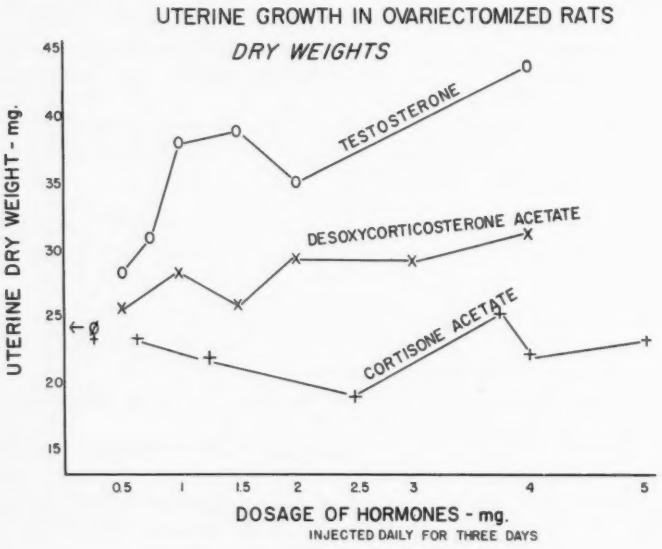

FIG. 2

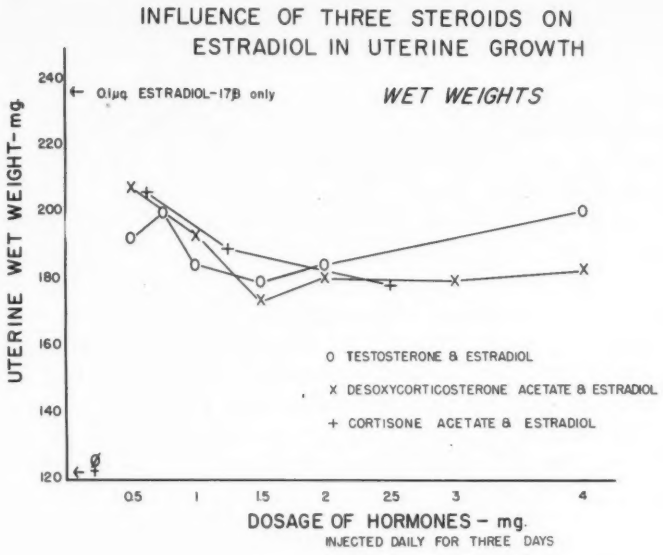

FIG. 3

When 4.0 mg. was given the average uterine weight was 262 mg. and the uteri were distended with fluid, an effect quite comparable to that produced by 0.1 μg. estradiol alone. These increases in weight of the uteri in response to testosterone are further reflected in the dry weights (Fig. 2).

The changes in uterine wet and dry weights produced by DCA were less

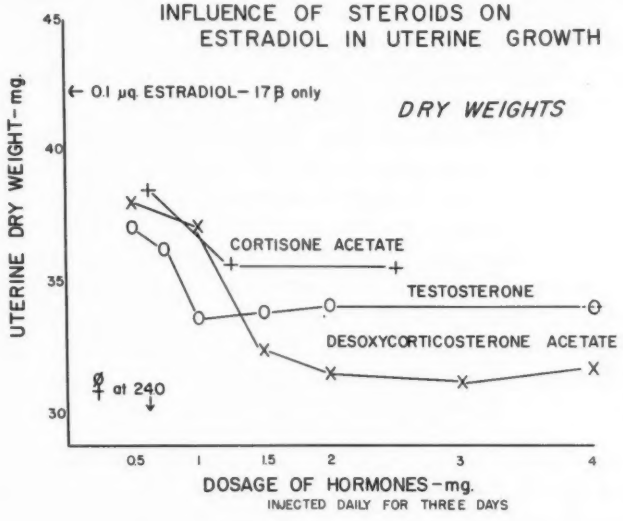

FIG. 4

striking than those for testosterone (Figs. 1 and 2). One-half mg. caused only a slight increase and larger doses brought about only a moderate increase.

Cortisone acetate did not cause appreciable growth of the uterus, in fact, those of animals given 0.5 to 2.5 mg. weighed less than those of castrated controls. Only doses of 3.75 mg. produced uterine weights that approached those of the control animals (Figs. 1 and 2).

These three steroids, when given concurrently in graded doses with a standard dose of 0.1 μg. estradiol, exercised a restrictive influence on the growth promoting action of the estrogen (Figs. 3 and 4). The inhibitory effects of each on increases in wet weight of the uterus in response to estradiol appeared about the same for doses up to 1.5 mg. while at 4.0 mg. DCA seemed more effective than testosterone. However, the dry weights of these uteri showed three distinct curves (Fig. 4). DCA seemed to be the strongest inhibitor at doses of 1.5 mg. and above while testosterone was the most effective when small doses were given. Cortisone acetate was the least effective of the three.

DISCUSSION

A point of interest brought out by these experiments is that cortisone acetate which produces little or no uterine growth even at high doses and desoxycorticosterone which is weak in this respect are nevertheless capable of restricting the growth promoting action of estradiol. This is in agreement with results obtained by Huggins and Jensen (5) in similar experiments with these two compounds. On the other hand, testosterone in doses of 4.0 mg. produced uteri of an average dry weight (43.6 mg.) equal to those for animals on 0.1 μg. estradiol (42.3 mg.) and yet when the two treatments were combined the uterine dry weight was only 34.1 mg. (Fig. 4). However, Huggins and Jensen (6) did not obtain inhibition of estrone by testosterone under their experimental conditions.

The inhibitory effects these three steroids have on uterine growth induced by estradiol are associated with differences in effect on the enzyme content of the uterus (1). Estradiol increases activity of the lactic dehydrogenase-DPNH oxidase system of the uterus by increasing DPNH oxidase while the other three steroids, like progesterone (2) tend to increase the lactic dehydrogenase component. Although the nature of such interactions is not understood, these and similar observations by other investigators do suggest the possibility that it may be an important feature of the normal physiology of steroid hormones.

SUMMARY

The comparative effectiveness of testosterone, desoxycorticosterone acetate and cortisone acetate in the promotion of uterine growth was determined by giving each in graded doses daily for three days to one-hun-

dred-day-old rats that had been castrated seven days previous to the beginning of treatment. It was found that testosterone was by far the most active, and that desoxycorticosterone acetate was relatively weak while cortisone acetate did not cause appreciable growth even at high doses. Each of the three steroids decreased the growth response when given concurrently with 0.1 μg. estradiol-17β. Testosterone was more effective at doses between 0.5 and 1.5 mg., desoxycorticosterone acetate more effective at doses of 1.5 to 4.0 mg., while the inhibition with cortisone acetate was less than that of the other two compounds.

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EFFECTS OF STRESS ON SERUM C^{14} LEVELS IN RATS FOLLOWING ADMINISTRATION OF HYDROCORTISONE-4-C^{14} AND CORTICOSTERONE-4-C^{14}

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ONE of the theories which has been proposed to explain the increased pituitary secretion of ACTH after the application of a stressful stimulus to the organism, is the concept that the increased demand of the peripheral tissues for adrenocortical hormones during stress results in a lowering of the cortical hormone titer in the blood, thereby stimulating the pituitary to secrete increased quantities of ACTH (Sayers 10).

In the experiments reported in this paper, C^{14} labelled hydrocortisone and corticosterone were used in rats in an attempt to determine whether the application of such procedures as exercise, the injection of epinephrine or histamine altered (a) the half-life of the radioactive label in the blood or (b) the blood levels of the radioactive steroid when it was infused into the animals at a constant rate.

If increased utilization, resulting in the destruction of the steroid ring, did occur then either the half-life of the compound would be decreased or, in the case where constant infusion was employed, the level of the blood steroid should decline.

METHODS

General.—Albino male rats of the Sprague-Dawley strain, weighing between 275 and 400 gm., were used. The animals were fed Purina laboratory dog chow *ad libitum* and had free access to water. Bilateral adrenalectomy was performed via the lumbar approach under ether anesthesia, and the rats, maintained on 0.9% NaCl solution, were used 3–4 days post-operative. Nephrectomized animals, operated via the abdominal route, were used immediately after operation.

Each rat was kept anesthetized during the entire period of the experiment. A 2% normal saline solution of sodium pentobarbital was administered intraperitoneally, in a dose of 4 mg./100 gm. of body weight to adrenalectomized animals, and 5 mg./100 gm. of body weight to all other animals.

Exercise.—Rats were exercised by inserting two electrodes into the thighs of each leg. The electrodes, made of fine copper wire soldered to steel sewing pins, were connected to a square wave stimulator, and stimulation was carried out at an intensity of

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either 10.5 or 21 volts, a frequency of 5 shocks/second, and a pulse duration of 1 millisecond.

Epinephrine.—Normal and adrenalectomized rats were injected subcutaneously with 20–40 μg./100 gm. body weight of a 0.02% normal saline solution of l-epinephrine hydrochloride which was prepared just before use. Eviscerated rats were infused with 2 μg. of l-epinephrine bitartrate/100 gm. body weight/hour via the femoral vein. The latter was cannulated with PE 50 polyethylene tubing and the infusion was made through a 22-gauge needle connected to a perfusion pump which was adjusted to deliver 0.54 ml. of fluid/hour. The epinephrine bitartrate was infused as a solution in a 0.0037% normal saline solution to which had been added 20 mg. of reduced glutathione /100 ml. saline to protect the epinephrine from oxidation during the infusion.

Histamine.—Intact rats were injected via the external jugular vein with 7.8 or 10 mg. of histamine dihydrochloride/100 gm. of body weight dissolved in a 10% solution of normal saline.

Infusion experiments.—A solution of either hydrocortisone-4-C^{14} or corticosterone-4-C^{14} was infused at a rate of 0.85 ml./hour via the external jugular vein which had been cannulated with PE 50 polyethylene tubing. The solution was prepared by adding 2 ml. of a 20% ethanol in normal saline solution of the radioactive steroid (1.6 microcuries) to 12 ml. of normal saline. At 15 minute intervals after the start of the infusion, approximately 0.3 ml. samples of blood were obtained from the tail and allowed to clot. It was then centrifuged at room temperature for 15 minutes at 2800 rpm. 0.1 ml. of serum was then placed on flat copper planchets (Tracerlab), air dried and the radioactivity determined using a windowless flow counter (Tracerlab). All samples were counted for 1024 counts, and since the amounts of serum on each planchet were identical, no corrections were made for self-absorption. The radioactivity of the samples is expressed as counts per minute per 0.1 ml. of serum above the background.

Eviscerated rats.—Evisceration was performed according to the method of Ingle (7) with the exception that asepsis was not maintained in either of the two stages of the operation. When adrenalectomy was also performed, the adrenals were removed after excision of the liver. Immediately following operation, each rat began receiving an infusion (0.85 ml./hour) via the external jugular vein of a mixture prepared in the following manner: 21 ml. normal saline solution, 4.06 gm. dextrose, 11,200 units buffered crystalline penicillin G, and 23 mg. streptomycin sulfate. 2½–3 hours after the start of the infusion, a similar solution was substituted which contained 18 ml. normal saline and 3 ml. of a 20% ethanol solution in normal saline of either hydrocortisone-4-C^{14} or corticosterone-4-C^{14} (2.4 microcuries). At 15 minute intervals following the start of the radioactive steroid infusion, approximately 0.3 ml. samples of blood were obtained from the carotid artery which had been cannulated with PE 50 polyethylene tubing just before evisceration. Blood was obtained in this manner because the blood flow in the tail of these eviscerated animals was so sluggish that it was virtually impossible to obtain a sufficient quantity in less than 4–5 minutes. The blood was processed and assayed as described above.

RESULTS

A. Half-time of C^{14} label in serum following intravenous injection of hydrocortisone-4-C^{14} and corticosterone-4-C^{14}.—In order to determine the half-time of the C^{14} label in the serum, rats were injected via the external jugular vein with 50 μg. of hydrocortisone-4-C^{14} or 50 μg. of cortisosterone-4-C^{14} (specific activity = 1.467 millicuries/m. mole) dissolved in 0.25 ml. 20% ethanol in normal saline solution. At intervals of 1, 3, 5, 7, 9, 11, 13, 15, 20, 30, 40, 50, and 60 minutes following administration of the radioactive ster-

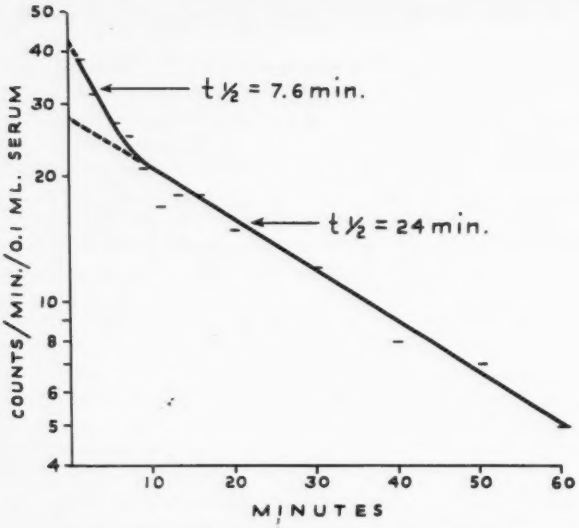

FIG. 1. Semilog plot of C^{14} label in serum, expressed as counts/min./0.1 ml. serum, *versus* time following the intravenous administration of 0.2 microcurie of corticosterone-4-C^{14} into a normal rat.

oid, approximately 0.3 ml. samples of blood were withdrawn from the tail in a period of time not exceeding 30 seconds. Figure 1 presents the results of a typical experiment using corticosterone.

When the radioactivity in counts/minute/0.1 ml. serum is plotted semilogarithmically against time, a curve is obtained which can be divided into two components: a rapid one with a half-time ($t_{\frac{1}{2}}$) of less than 10 minutes and a slower one with a $t_{\frac{1}{2}}$ of 20–30 minutes. Similar results were obtained with hydrocortisone-4-C^{14}.

TABLE 1. HALF-TIMES OF C^{14} LABEL IN SERUM OF RATS FOLLOWING INTRAVENOUS ADMINISTRATION OF HYDROCORTISONE-4-C^{14} AND CORTICOSTERONE-4-C^{14}

	Hydrocortisone		Corticosterone	
	Rapid component minutes	Slow component minutes	Rapid component minutes	Slow component minutes
Normal	9.7 ± 0.8* (10)†	26 ± 1.6 (7)	9.6 ± 0.7 (9)	18.7 ± 0.9 (9)
Normal + exercise	8.4 ± 0.5 (9)	23 ± 1.5 (3)	7.7 ± 0.9 (7)	19.4 ± 1.1 (7)
Adrenalectomized control	7.3 ± 0.4 (10)	31 ± 1.2 (9)	—	—
Adrenalectomized + exercise	7.4 ± 0.4 (11)	32 ± 3.4 (6)	—	—

* Mean half-time ± standard error.
† Number of animals in each group.

It can be seen from the data in Table 1 that although the $t_{\frac{1}{2}}$ of the rapid component of the hydrocortisone and corticosterone curves are similar, the $t_{\frac{1}{2}}$ of the slow component of the corticosterone curve is significantly shorter ($P < .01$). Exercise appeared to have no effect on the $t_{\frac{1}{2}}$ of the C^{14} label after the injection of either of the two radioactive steroids.

Adrenalectomy decreased the $t_{\frac{1}{2}}$ of the rapid component of the hydrocortisone curve ($p < .02$), whereas it increased the $t_{\frac{1}{2}}$ of the slower one ($P < .02$). Exercise again did not alter these values.

B. Intravenous infusion of hydrocortisone-4-C^{14} and corticosterone-4-C^{14} corticosterone into non-eviscerated rats.—In Table 2 are combined the results of the infusion of labelled hydrocortisone or corticosterone into normal or adrenalectomized rats. As described above, blood was collected for measurement of the serum radioactivity every 15 minutes from the start of the continuous intravenous injection of the steroid. When no other procedure other than the infusion is applied to the animals, the radioactivity of the serum remains constant after the beginning of the injection from the fifth to the twelfth 15 minute period. In subsequent experiments (Table 2) where a stress was applied during the infusion, the beginning of the stress is indicated by the heavy vertical bars.

These results show that exercise, the subcutaneous injection or intravenous infusion of epinephrine or the intravenous infusion of histamine do not decrease the level of radioactivity in the serum of either normal or adrenalectomized animals. The infusion of corticosterone, the characteristic adrenal steroid in the rat, does not show any differences from those observed with hydrocortisone. In the case of both steroids there is no evidence, at least by this technique, that the procedures employed increased the rate of disappearance of the label from the blood. Removal of the kidneys immediately before the infusion also did not cause any change in the rate of disappearance of the label when stress was applied to the animals.

In some experiments (Table 2, Line D and G) the level of serum radioactivity rose significantly after the application of the stress. This may indicate that the procedure employed in these instances, intravenous infusion of histamine or epinephrine, caused sufficient circulatory difficulties to retard the rate of removal of the labelled compound.

C. Intravenous infusion of hydrocortisone-4-C^{14} and corticosterone-4-C^{14} into eviscerated rats.—Figure 2 presents the results of a typical experiment in which corticosterone-4-C^{14} was infused into an eviscerated rat for a period of 4 hours. In contrast to the plateau found in intact animals (Table 2) the level of radioactivity of the serum rises continuously throughout the period of infusion. Furthermore, it increases at a constant rate from the very beginning of infusion, a rate that remains constant throughout the period studied. Similar curves were obtained when C^{14}-hydrocortisone was infused.

Exercise or the intravenous infusion of epinephrine, begun 1 hour after the beginning of the infusion of labelled hydrocortisone or cortiscoterone

TABLE 2. EFFECT OF EXERCISE, EPINEPHRINE AND HISTAMINE ON THE SERUM RADIOACTIVITY (COUNTS PER MINUTE PER 0.1 ML.) OF NORMAL AND ADRENALECTOMIZED RATS INFUSED WITH HYDROCORTISONE-4-C^{14} AND CORTICOSTERONE-4-C^{14}. THE BLACK VERTICAL BARS INDICATE THE TIME AT WHICH THE STRESS WAS APPLIED

Group no.	C^{14} steroid infused	Animals		Stress applied	Period								
		type	No.		4	5	6	7	8	9	10	11	12
A	Hydrocortisone	N	4	None	6±0.2	9±0.9	9±1.6	9±0.7	10±0.4	10±1.4	10±0.5	10±1.2	12±0.9
B	Hydrocortisone	N	5	Exercise	12±0.6	13±0.5	14±1.0	14±1.4	14±0.7	15±1.1	16±0.6	17±2.2	20±5.5
C	Hydrocortisone	N	8	Epinephrine S.C.	13±0.6	12±0.7	13±0.8	14±0.7	14±0.7	13±0.4	14±0.8	14±0.6	14±0.5
D	Hydrocortisone	N	3	Histamine I.V.	14±0.7	15±0.9	15±0.4	17±0.5	17±2.1	19±1.3	19±0.9	20±1.2	18±1.2
E	Hydrocortisone	Ad.	7	Epinephrine I.V.	20±0.5	20±1.8	21±1.2	22±0.8	23±2.0	26±1.5	27±1.2	26±1.0	27±1.4
F	Corticosterone	N	4	Exercise	9±0.6	10±1.1	10±1.1	10±1.0	10±1.2	10±2.4	12±1.9	13±3.9	10±0.9
G	Corticosterone	N	5	Epinephrine I.V.	11±1.2	11±0.7	14±1.4	15±1.5	15±1.4	15±1.2	15±1.3	15±1.9	15±1.6

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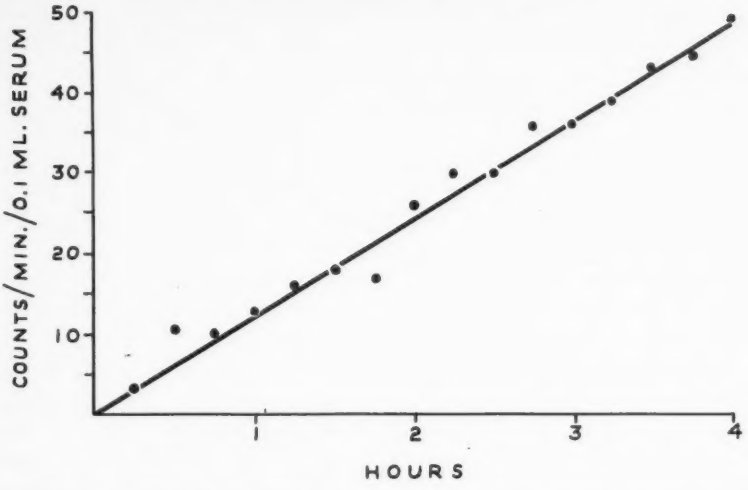

FIG. 2. Serum C^{14} levels, expressed as counts/min./0.1 ml. serum, during the continuous intravenous infusion of corticosterone-4-C^{14} into an eviscerated rat.

and continued for the duration of the experiment, did not change the slope of the curve (Figs. 3 and 4).

In some experiments these stresses were applied to the animals 1 to 2 hours before the infusion began and were continued throughout. Again there was no significant difference in the slopes of the serum C^{14} curves. In a few experiments adrenalectomized eviscerated animals were used but the

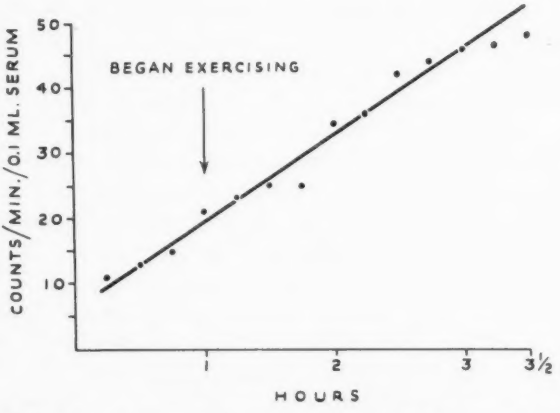

FIG. 3. Serum C^{14} levels, expressed as counts/min./0.1 ml. serum, during the continuous intravenous infusion of hydrocortisone-4-C^{14} into an eviscerated rat which was exercised beginning at 1 hour.

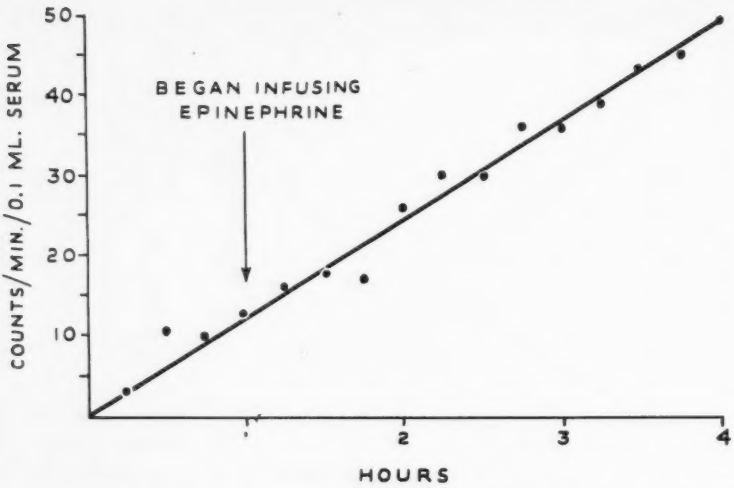

FIG. 4. Serum C^{14} levels, expressed as counts/min./0.1 ml. serum, during the continuous intravenous infusion of hydrocortisone-4-C^{14} into an eviscerated rat which began receiving an intravenous infusion of l-epinephrine at 1 hour.

results were no different from those obtained with non-adrenalectomized eviscerated animals.

DISCUSSION

The results of the experiments reported in this study lead to the conclusion that such stresses as exercise, epinephrine or histamine do not result in an increased rate of utilization of adrenocortical hormones by the rat. However, it is necessary to point out several features of these experiments that still leave doubt as to their finality in the acceptance of this conclusion.

In the first place only the C^{14} content of the serum was measured and not the specific activity of the isolated steroids. Since 0.1 ml. samples of serum were analyzed for radioactivity, present techniques for isolating and measuring blood steroids would not be adequate. Nevertheless, if the label remained in the steroid nucleus it can be estimated from the specific activity of the steroid administered (3000 counts/min./microgram) and the activity measured in 0.1 ml. of serum, that quantities of the order of 0.003 μg. of steroid were detectable. Such quantities are far below those that can be measured by the most sensitive chemical techniques (2).

Recent experiments by Abelson, Ulrich, and Long (1) throw some light on the question of what steroids are being measured in the serum of the rat following the administration of hydrocortisone-4-C^{14} and corticosterone-4-C^{14} when C^{14} determinations are carried out. These investigators showed that after the intravenous administration of hydrocortisone into normal rats, 20β-hydroxyhydrocortisone (Reichstein's Compound E) was the substance detected in most abundance in plasma 7 minutes after injec-

tion. Already 1 minute after injection a considerable amount of 20β-hydroxyhydrocortisone was found in the plasma. Since there was very little C^{14} activity in the non-chloroform extractable portion of the plasma following the intravenous administration of hydrocortisone-4-C^{14} (Abelson, Ulrich and Long, unpublished observations), it would appear that within the first few minutes following injection, primarily unconjugated hydrocortisone and 20β-hydroxyhydrocortisone were being measured.

Although it appears that in normal animals either injected or infused with hydrocortisone-4-C^{14}, the C^{14} represents primarily 20β-hydroxyhydrocortisone, this does not appear to be so with corticosterone. Unpublished experiments by Abelson, Ulrich, and Long show that when corticosterone-4-C^{14} was injected intravenously into normal rats, corticosterone was the principal steroid detected in the plasma up to 30 minutes following injection. Furthermore, there was very little radioactivity present in the non-chloroform extractable portion of the plasma.

The C^{14} measured in the serum of eviscerated rats infused with hydrocortisone-4-C^{14} seems to represent mainly hydrocortisone since Abelson, Ulrich, and Long (1) demonstrated that there was very little conversion of hydrocortisone to 20β-hydroxyhydrocortisone and other steroids at 1, 7, 15, and 30 minutes following the intravenous injection of hydrocortisone-4-C^{14} into such animals.

A second criticism of these experiments is that the level of radioactivity in the serum was so low that the error in counting the C^{14} was of the order of 10 to 15%. This was especially so in the experiments in which radioactive hydrocortisone and corticosterone were infused into non-eviscerated rats. Here the radioactivity in 0.1 ml. of serum was usually between 10 and 20 counts per minute above background. In view of the fact that any effects of stress on the serum C^{14} levels were of doubtful significance, it might well be asked whether any small, significant changes could be detected at such low levels of radioactivity.

However, in the eviscerated animals where the serum radioactivity was several times as large no effect of the stress was observed.

Because the data obtained from these experiments were mainly negative, the question arises as to whether the intensity of the stressors was sufficient to cause a possible increased utilization of hydrocortisone and corticosterone. This can not be answered, since if negative results were obtained after a stress of still greater intensity, it might well be asked again whether the stress was great enough.

It is conceivable that if a different type of stressor were used some evidence for increased utilization of adrenocortical hormones might have been found. In this study exercise and epinephrine were used because they were considered to be physiological stressors which would not cause temporary or permanent damage to tissues, in contrast to such pathological stressors as burns or the injection of toxic substances. In this connection it

should be pointed out that the stress of evisceration may have overshadowed the subsequent stress of exercise or epinephrine, despite the fact that at least 2½–3 hours were allowed to elapse between the end of the operation and the beginning of the radioactive steroid infusion.

Even though evisceration was undoubtedly a severe stress, this operation was performed for the following reasons. In the first place, changes in blood flow resulting from circulatory changes in the splanchnic area during stress may have been great enough to cause alterations in the peripheral circulation and thus mask any true changes in blood steroid levels. Secondly, since the liver has been shown to be one of the principal organs concerned in the metabolism of steroids (9), it was felt that an animal preparation consisting essentially of skeletal muscle might be more likely to show evidence for increased cortical hormone utilization during stress than one in which the liver was intact. This is especially so in view of the observation that liver function is depressed during surgery and other forms of stress (12). Brown, *et al.* (3), who observed increases in the plasma 17-hydroxycorticosteroid levels after surgery, suggested that this was the result of both increased adrenocortical hormone secretion and impaired removal of these steroids by the liver.

Another criticism of these experiments is that the doses of radioactive steroid administered (50 μg. in the "disappearance" experiments, and approximately 30 μg./hour in the infusion experiments) may not have been "tracer" amounts. When estimates of the daily production of cortical hormone were made by indirect measurements using adrenalectomized rats, values ranging from approximately 80 to 400 μg. of cortisone/350 gm. of body weight were observed (10). During such stresses as forced muscular work, the intraperitoneal injection of glucose, and exposure to an altitude of 20,000 ft., these values increased to between 1 and 7 mg. of cortisone/350 gm. of body weight. However, the amounts of radioactive steroid administered may not have been critical because Hellman, *et al.* (6) did not find any difference in the fate of a tracer dose of hydrocortisone administered to an adrenalectomized oophorectomized subject deprived of all therapy and a tracer dose of the hormone administered to the same patient while he was maintained on adequate amounts of intravenously administered hydrocortisone.

Some of the data in the literature on the effects of stress on the utilization of 17-hydroxycorticosteroids and their levels in the blood are contradictory. Staehelin, *et al.* (11) observed an increase in the plasma corticosteroids of human subjects 15 and 30 minutes after the start of muscular exercise, whereas at 1 and 2 hours the plasma steroid level had decreased. These investigators also found that when Addisonian patients given an infusion of cortisone were performing muscular exercise, a more rapid lowering of the plasma corticosteroid level occurred than when the patients remained in bed. In another study using dogs which were subjected to a

temperature of 5° C for a period of 6 hours, Eik-Nes (5) did not observe any increase in the levels of plasma 17-hydroxycorticosteroids above that seen in controls. Hydrocortisone, administered intravenously to such animals, was removed from the circulation at a reduced rate.

Cowie *et al.* (4) found a greater rather than a smaller degree of eosinopenia following small doses of hydrocortisone in adrenalectomized dogs subjected to trauma, indicating a smaller rather than a greater utilization of the hormone. A more direct approach to the problem was the work of Perrini, *et al.* (8) who studied the rate of reduction of the conjugated unsaturated system and side chain of cortisone acetate by liver slices. They found that normal rat liver slices were more active in this respect than slices of previously stressed rats.

The results of the experiments reported in this study appear to indicate that the application of such stressors as muscular exercise and epinephrine do not result in an increased rate of utilization of adrenocortical hormones by the rat. It should be pointed out, however, that blood corticoid levels may not truly reflect the metabolic changes that are occurring in the peripheral tissues of the stressed organism, since if an increased rate of utilization does occur it may be "1) shared by all cells of the organism, 2) involve only those cells affected by the stress stimulus, or 3) is participated in by both 'injured' cells and buffer organs attempting to restore homeostasis" (10). Furthermore, the term "utilization of adrenocortical hormones" could refer to one or a number of processes occurring in the peripheral tissues, such as enzymatic degradation of the hormone to functionally inactive metabolites, binding of the hormone by intracellular particles, transport of the hormone across cell membranes, etc. It is thus essential to study the metabolic fate of adrenocortical hormones in the peripheral tissues before one can really say whether or not increased utilization of cortical hormones occurs during stress.

SUMMARY

The C^{14} label of intravenously administered hydrocortisone-4-C^{14} and corticosterone-4-C^{14} disappeared from the serum of normal and adrenalectomized rats at a rate which could be characterized by an initial rapid component with a half-time of between 7 and 10 minutes and a slow component with a half-time of between 19 and 32 minutes. The half-times were not altered by electrically stimulating the animals to do muscular exercise.

When normal and adrenalectomized rats were given a continuous intravenous infusion of hydrocortisone-4-C^{14} or corticosterone-4-C^{14} for 3 to $3\frac{1}{2}$ hours and subjected to the stress of muscular exercise, epinephrine, or histamine at $1\frac{1}{2}$ to 2 hours after the start of the infusion, the level of C^{14} in the serum appeared not to be affected.

The slopes of the serum C^{14} curves of eviscerated rats given a continuous intravenous infusion of hydrocortisone-4-C^{14} or corticosterone-4-C^{14} did not

appear to change when such stressors as muscular exercise or epinephrine were applied either before or after the start of the radioactive steroid infusion.

Although little, if any, positive evidence was found to substantiate the concept of increased peripheral utilization of adrenocortical hormones during stress, the evidence obtained from these experiments would seem to warrant a conclusion of "not proven" as regards this theory.

Acknowledgments

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PARATHYROID FUNCTION AND THE PLASMA CITRIC ACID AND CALCIUM RESPONSE TO NEPHRECTOMY[1]

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THE relation of calcium and citric acid metabolism has been the subject of numerous studies, particularly since Dickens (1) demonstrated that the skeleton is rich in citric acid. However, it has remained a question as to whether or not there is a specific endocrine mechanism relating calcium and citric acid. Evidence presented previously from this laboratory in studies on adult dogs has shown that the hypercitricemia following nephrectomy is largely or entirely abolished if the thyroparathyroid apparatus is removed three weeks prior to nephrectomy (2). Further, that restoration of the serum calcium value of the thyroparathyroidectomized animal to normal by Vitamin D administration, did not restore the plasma citric acid response to nephrectomy. These observations suggest that increased concentration of citric acid in the circulation following nephrectomy depends specifically upon the function of the parathyroid gland.

The present study was undertaken to extend these observations to other species and to determine the effect of parathyroid hormone replacement on the response of parathyroidectomized rats to nephrectomy. The effect of Vitamin D on the response to nephrectomy was extended to include the rat. Experiments were planned to provide information on the following aspects of this problem:

1. The plasma calcium and citric acid response to nephrectomy of various species of animals.
2. The effect of parathyroidectomy prior to or simultaneous with nephrectomy on the plasma calcium and citric acid response.
3. The effect of administration of sufficient parathyroid extract[3] or Vitamin D to restore the serum calcium to normal on the response of the parathyroidectomized animal to nephrectomy.

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EXPERIMENTAL PROCEDURE AND METHODS

These observations were made on Sprague-Dawley rats weighing 350–400 gm., maintained on a diet of Rockland rat pellets. These animals were divided into groups of 10 rats each and fasted for 12 hours prior to bleeding or nephrectomy. All bleedings were from the abdominal aorta, while the animal was lightly anaesthetized with ether. Blood was collected in a 20 cc. syringe containing 0.1 ml. of heparin and all analyses were made on individual samples of freshly separated plasma. The methods of analysis have been indicated previously (3).

The parathyroid glands of the rat were destroyed by direct application of an electrical cautery to the gland visualized with the aid of low-power binoculars. The upper pole of the thyroid gland was likewise destroyed by cautery. Parathyroidectomy in the rabbit was by surgical means and in all other species the entire thyroparathyroid apparatus was removed. Aseptic surgical technique was adhered to in all operations save those on the rat. Nephrectomy, ureteral ligation and splenectomy were through a midline abdominal incision.

RESULTS

First, a comparison was made of the plasma citrate and calcium in normal, ureteral ligated, splenectomized and nephrectomized rats (Fig. 1).

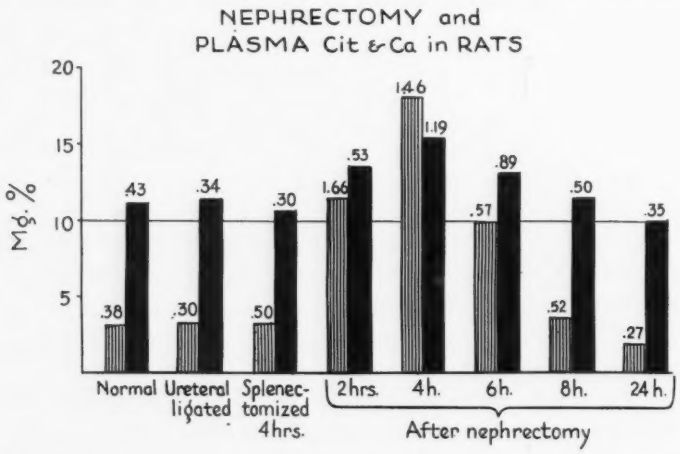

FIG. 1. Presenting a comparison of normal plasma values for calcium and citric acid with results obtained 4 hours after ureteral ligation or splenectomy and at various intervals after nephrectomy. Calcium concentration indicated by the solid column. There were 10 rats in each group. Standard deviation of the individual values for each group is indicated by number at top of each column.

It was found that only nephrectomy produced a significant effect on the plasma values as compared to the normal group (Table 1). Comparison of plasma values at 2-hour intervals following nephrectomy demonstrated a maximum increase in plasma citrate and calcium 4 hours after nephrectomy with a return to normal range after 8 hours (Fig. 1). Thereafter, all

TABLE 1. COMPARISON OF PLASMA CALCIUM AND CITRIC ACID VALUES OF EXPERIMENTAL GROUPS OF RATS AND THEIR CONTROLS*

Groups† compared	Per cent probability		Groups† compared	Per cent probability	
	Calcium	Citric acid		Calcium	Citric acid
1– 2	>.05	>.05	6– 7	>.05	>.05
1– 3	>.05	>.05	6– 8	>.05	>.05
1– 4	<.01	<.01	6– 9	<.01	<.01
1– 5	<.05	<.01	6–11	<.01	<.01
1– 6	<.01	<.01	6–15	<.01	<.01
1– 9	>.05	>.05	8–10	<.01	<.01
1–11	<.01	>.05	8–12	<.01	<.01
1–13	<.01	<.01	8–16	<.01	<.01
4– 5	<.01	<.01	9–11	<.01	>.05
4– 8	<.01	<.01	9–15	>.05	<.01
4–10	>.05	>.05	10–12	<.01	<.01
4–12	<.01	<.01	10–16	<.01	<.01
4–14	>.05	>.05			

* All bloods drawn 4 hours after nephrectomy or comparable procedure unless otherwise stated.

† (1) Normal, (2) Ureteral Ligated, (3) Splenectomized, (4) Nephrectomized, (5) Parathyroidectomized and Nephrectomized, (6) Parathyroidectomized, (7) Parathyroidectomized after 4 days splenectomized, (8) Parathyroidectomized, after 4 days nephrectomized, (9) Parathyroidectomized, parathyroid extract injected daily over 4 day period, (10) Parathyroidectomized, parathyroid extract injected daily over 4 day period, nephrectomized on 4th day, (11) Parathyroidectomized, single injection parathyroid extract 3rd day, (12) Parathyroidectomized, single injection parathyroid extract on 3rd day, nephrectomized on 4th day, (13) Vitamin D daily for 7 days, (14) Vitamin D daily for 7 days, nephrectomized on 8th day, (15) Vitamin D daily for 7 days, parathyroidectomized 4th day on Vitamin D, bled on 8th day, (16) Vitamin D daily for 7 days, parathyroidectomized 4th day on Vitamin D, nephrectomized on 8th day.

comparisons on various groups of rats were made on blood samples drawn 4 hours after nephrectomy.

The effect of parathyroidectomy on the response of the rat to nephrectomy was then studied. One group of rats was parathyroidectomized and nephrectomized at the same operation and bled 4 hours later. There was a significant increase in plasma citrate, but only a slight change in the calcium values as compared to the control groups (Fig. 2 and Table 1). Another group of rats parathyroidectomized 4 days prior to nephrectomy showed no change in plasma citrate and calcium values following nephrectomy as compared to parathyroidectomized groups that were not nephrectomized or in which splenectomy was performed rather than nephrectomy (Fig. 2). These results were consistent and gave highly significant differences as compared to the response of the normal rat to nephrectomy (Table 1).

The effect of parathyroid extract on the response to nephrectomy of the parathyroidectomized rat was determined next. Two groups of rats were parathyroidectomized and given 30 I.U. of parathyroid extract the day of parathyroidectomy and twice daily for the next three days, a total of 210 I.U. On the 4th day following parathyroidectomy, one group was bled and the other group was nephrectomized and bled 4 hours later. The results indicate that the parathyroid extract had maintained the plasma calcium

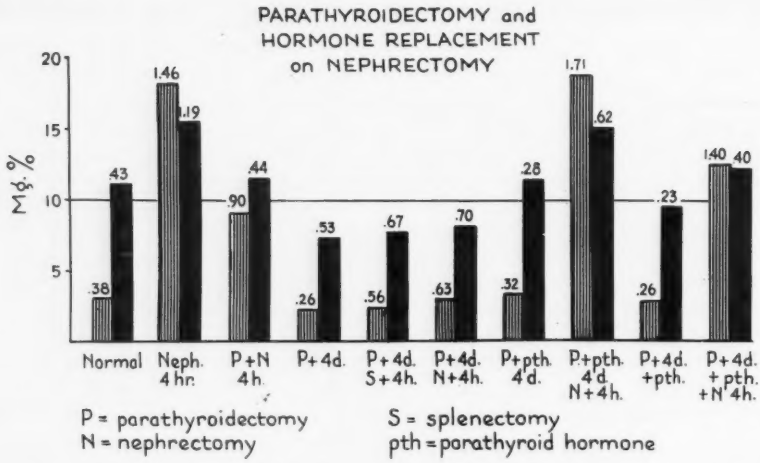

FIG. 2. Showing the effect of parathyroidectomy and of parathyroid extract on the plasma calcium and citric acid response to nephrectomy of adult rats. Each column indicates the average of individual values obtained on 10 rats. Standard deviation of values indicated by number at top of column. Solid columns represent calcium.

Neph.-4 hr. (Nephrectomized and bled after 4 hours.)

P+N (Parathyroidectomized and nephrectomized at same operation—bled after 4 hours.)

P+4d (Parathyroidectomized and bled 4 days later.)

P+4d S+4 hrs. (Parathyroidectomized, 4 days later splenectomized and bled 4 hours following splenectomy.)

P+4d N+4h (Parathyroidectomized, 4 days later nephrectomized and bled 4 hours after nephrectomy.)

P+pth 4d (Parathyroidectomized, given parathyroid extract daily and bled on 4th day post op.)

P+pth 4d N+4h (Parathyroidectomized, given parathyroid extract daily, nephrectomized on 4th day following parathyroidectomy and bled 4 hours following nephrectomy.)

P+4d+pth (Parathyroidectomized, given a single large dose of parathyroid extract on the 3rd day post op. and bled on the 4th day post op.)

P+4d+pth+N4h (Parathyroidectomized, given a single large dose of parathyroid extract on the 3rd day post op., nephrectomized on the 4th day post parathyroidectomy and bled 4 hours after nephrectomy.)

and citric acid in normal range and that nephrectomy was followed by a rise in plasma citrate and calcium of similar magnitude to that obtained in normal animals following nephrectomy (See Fig. 2 and Table 1).

The effect of a single large dose of parathyroid extract on the response to nephrectomy was also determined. Two groups of rats were parathyroidectomized and 3 days later each rat was injected subcutaneously with 200 I.U. of parathyroid extract. Sixteen hours later one group was bled and the other group was nephrectomized and bled after 4 hours. Comparison of

the plasma values of these groups indicates that the single large dose incompletely restored the plasma values to normal and that the increase in calcium and citric acid following nephrectomy, though definite, was significantly less than when the hormone had been administered in divided doses daily following removal of the parathyroid gland (Fig. 2 and Table 1).

To determine the effect of Vitamin D on the response to nephrectomy of normal and parathyroidectomized rats, four groups of rats were given 5000 I.U. Vitamin D daily orally for 7 days. Two groups were parathyroidectomized the 4th day on Vitamin D. On the 8th day, one intact and one parathyroidectomized group were nephrectomized and bled 4 hours later,

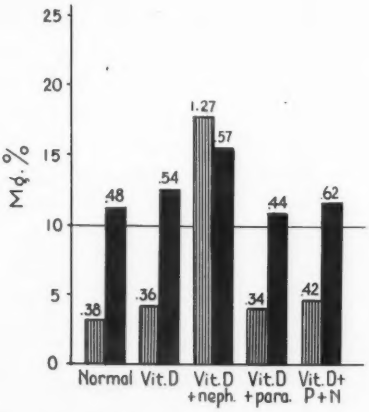

FIG. 3. Showing the effect of Vitamin D on the response of normal and parathyroidectomized animals to nephrectomy. Each column represents average values on groups of 10 rats. Standard deviation of values indicated by number at top of column. Solid column indicates plasma calcium values.

Vit. D (Normal rats given 5000 I.U. Vitamin D daily for 7 days.)

Vit. D+Neph. (Normal rats given 5000 I.U. of Vitamin D daily for 7 days, nephrectomized on the 8th day and bled 4 hours later.)

Vit. D+para. (Rats given 5000 I.U. Vitamin D daily for 7 days, parathyroidectomized on 4th day of Vitamin D administration and bled 4 days after parathyroidectomy.)

Vit. D+P+N (5000 I.U. Vitamin D daily for 7 days, parathyroidectomy on 4th day of Vitamin D administration, nephrectomized 4 days after parathyroidectomy and bled 4 hours later.)

while the other two groups were merely bled. The values obtained on the different groups, shown in Figure 3, indicate that the amount of Vitamin D administered caused a slight elevation of the plasma values of the intact animals and maintained the plasma values in the normal range following parathyroidectomy. However, the response to nephrectomy in the intact and lack of response in the thyroparathyroidectomized animals were not modified by Vitamin D administration.

The effect of nephrectomy on the plasma calcium and citrate was likewise studied in the guinea pig, rabbit, baboon and monkey. Twelve adult guinea pigs were divided into two equal groups. Six animals were bled 4 hours after nephrectomy and the values compared with the control group. Results on the two groups, shown in Figure 4, indicate a clearly defined hypercalcemia and hypercitricemia following nephrectomy. The citric acid elevation was greater than that observed in the rat.

Normal plasma values were obtained on 14 large domestic rabbits. Seven of these animals were nephrectomized and bled at various intervals up to 24 hours. The peak in the plasma increase in citric acid and calcium

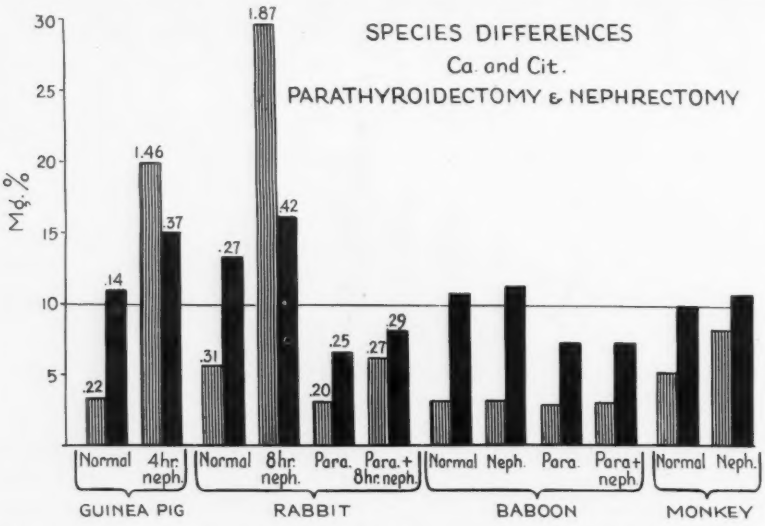

FIG. 4. Presents data showing the species difference in response of the plasma calcium and citric acid to nephrectomy. The effect of parathyroidectomy is also demonstrated for the rabbit and baboon.

occurred 8 hours after the kidneys were removed. The maximal elevation in citric acid was clearly in excess of that observed in the other species studied (Fig. 4). Seven rabbits were parathyroidectomized 4 days prior to nephrectomy and were bled before and 8 hours after nephrectomy. Removal of the kidneys was followed by a slight increase in the plasma concentration of calcium and citric acid, but the response was much smaller than that of the group having parathyroid glands (Fig. 4). Observations were made on 4 healthy male dog-faced baboons. Nephrectomy produced no effect on the plasma values of two animals 4, 8 or 12 hours after the operation (Fig. 4). The plasma values of two thyroparathyroidectomized baboons were likewise unaffected by nephrectomy. Two healthy Rhesus monkeys, bled before and at 4 hour intervals for 12 hours after nephrectomy, showed only a slight increase in plasma calcium and citric acid (Fig. 4).

DISCUSSION

The results presented demonstrate clearly that the parathyroid gland exerts an effect on the plasma calcium and citric acid response to nephrectomy. It seems evident that the transient hypercalcemia occurring in some species of animals following nephrectomy is due to the increased concentration of citric acid in the circulation. In no instance has a hypercalcemia following nephrectomy occurred in the absence of a hypercitricemia. However, some degree of hypercitricemia has been observed in the absence of an hypercalcemia, for example, in the group of rats that were parathyroidectomized and nephrectomized at the same operation (Fig. 2). Hence, the change in citric acid metabolism is probably the primary effect so far as the influence of nephrectomy on the plasma concentration of these two substances is concerned.

The accumulation of citric acid in the circulation following nephrectomy must be due to a disproportion between the rate of mobilization of citric acid into the circulation and the rate of removal or destruction by the tissues of the body. Martensson (4) demonstrated the marked capacity of the perfused kidney to destroy citric acid. This author also demonstrated a marked rise in the plasma citric acid of rats and rabbits following nephrectomy and that the rise was much less in cats. Published (3) and unpublished (5) data from this laboratory have shown that there is a marked reduction in the rate of removal of injected citric acid from the circulation of the nephrectomized dogs. This change in the removal of citrates from the circulation must be largely dependent upon the metabolism of the kidney rather than a failure in excretion, since the ureteral ligated rat does not get a hypercitricemia (Fig. 1), neither does the ureteral ligated dog (3) and the last mentioned preparation shows only a slight reduction in the rate of removal of injected citrate as compared to the normal animal (5).

The effect of nephrectomy on the plasma citrate concentration is a transient phenomenon, which can only be demonstrated by collecting blood samples at appropriate intervals. Apparently, the increase begins soon after nephrectomy, since an elevation in plasma citric acid was clearly apparent in the rat 2 hours after nephrectomy (Fig. 1) and also has been demonstrated this early in nephrectomized dogs (5). The return of the plasma citrate value to the normal range was complete in the rat after 8 hours and usually in the dog 24 to 48 hours after nephrectomy. The serum calcium returns to normal before or in association with the reduction of circulating citrates. Vitamin D does not appear to affect the response of the animal to nephrectomy regardless of whether the parathyroid gland is present or absent. The results with Vitamin D help to exclude the calcium concentration of plasma as a factor in the citrate response to nephrectomy, since the hypoparathyroid Vitamin D treated animals had normal plasma calcium values yet behaved like the hypocalcemic parathyroidectomized animals following nephrectomy.

This finding suggests that Vitamin D and parathyroid hormone do not have a similar action on mechanisms affecting citric acid mobilization or metabolism. At the present time, there is no adequate basis for deciding what may be the source or fate of circulating citrates. Undoubtedly, there is a continuous turnover of citrates between the circulation and certain tissues of the body. Based on citric acid balance and bone analysis, Class and Smith (6) concluded that the excess of citric acid excreted by the kidney after sodium bicarbonate or sodium malate does not originate in the skeleton, but is a product of intermediary metabolism. The use of tagged citric acid may help to elucidate the extent of turnover of circulating citrates. Temporary disturbance in the balance of this process resulting from removal of the kidneys followed by a rapid return to normal concentration suggests that other tissues increased their rate of citrate removal from the circulation following nephrectomy or that nephrectomy causes a transient increase in the rate of citrate mobilization. Failure to demonstrate any change in circulating citrates following nephrectomy in the parathyroidectomized animal could be due to a reduced turnover of citrate or to the decrease or absence of a factor regulating the extent of citrate uptake or destruction by certain tissues of the body. The lack of effect of nephrectomy on the plasma calcium and citric acid of baboons and monkeys suggests that the removal of citrates from the circulation is less dependent on the kidney in these species.

There is no adequate basis at present for deciding to what extent the citrates in bone may play an active role in the metabolism of this tissue. Steenbock and coworkers (7, 8) have shown an association between calcification of bone and citrate content as influenced by Vitamin D in rachitic animals. It has likewise been shown that whenever calcification occurs in the living organism, citrate accumulation also occurs. Kuyper (9) has shown that citrate precipitates in definite proportion if it is present when calcium phosphate is formed. The low dissociation constant of calcium citrate complex may explain the elevated plasma content of calcium in the presence of a high concentration of circulating citrates and calcium may in turn stabilize citrate and prevent its destruction. However, the physical-chemical interrelation does not adequately account for the difference in the response of the normal and parathyroid insufficient animal to nephrectomy. The most plausible explanation appears to be that parathyroid hormone has some direct effect on citric acid metabolism.

SUMMARY

Nephrectomy produced a transient hypercitricemia and hypercalcemia in the rat, rabbit and guinea pig, but not in the baboon and monkey.

Parathyroidectomy 4 days prior to nephrectomy abolished the transient hypercitricemia and hypercalcemia that followed nephrectomy in the normal rat and rabbit.

Parathyroid extract restored the calcium and citric acid response of the parathyroidectomized rat to nephrectomy. Vitamin D in amounts adequate to restore the plasma calcium values failed to restore the response of the parathyroid insufficient animal to nephrectomy.

Evidence is summarized pointing to an effect of parathyroid hormone on citric acid metabolism in the rat, rabbit and guinea pig.

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THE EFFECT OF FLUOROACETATE UPON THE PLASMA CITRATE RESPONSE TO PARATHYROIDECTOMY AND NEPHRECTOMY[1]

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THE regulating mechanism by which the parathyroid gland maintains the concentration of ionized calcium in the body and the extent to which the kidney may contribute to this process is not understood. Some insight into the metabolism and turnover of circulating citrates is essential to determine any possible role this substance might have in mobilizing calcium from the skeleton or in stabilizing the calcium ion content of body fluids. Preceding experiments (1, 2) indicate that the balance between mobilization and removal of citrates from the circulation is temporarily affected by nephrectomy and that parathyroidectomy abolished the evidence of imbalance produced by nephrectomy.

The observation by Buffa and Peters (3) and others (4, 5) indicates that fluoroacetate blocks the destruction of citric acid in most tissues excepting the liver. The present experiments were planned to demonstrate the effect of blockade of the citric acid cycle upon the concentration of citric acid and calcium in the circulation. By applying this blockade to animals representing all the combinations of conditions previously considered (1) it was hoped that some further appreciation of factors concerned in the turnover of circulating citrates might be obtained.

EXPERIMENTAL PROCEDURE AND RESULTS

The rats, as well as the laboratory and surgical procedures employed for this study, were as described in the preceding article (1). Each group contained 10 rats. Sodium fluoroacetate was freshly dissolved for each experiment and injected intraperitoneally 6 hours before an animal was bled. The total dose for each rat was administered in 2 ml. of water and was generally 5 mg./Kg., although 2.5 mg./Kg. was used in some experiments.

Groups 1, 2, 3, 5, 12 and 14 from the previous study are included with the data for comparison.

Normal rats injected intraperitoneally with either dose of fluoroacetate and bled 6 hours later showed a significant rise in plasma citric acid and a

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slight increase in the calcium concentration (Tables 1 and 2, Groups 2 and 9). Rats injected with 5 mg./Kg. fluoroacetate, nephrectomized 2 hours later and bled 4 hours after nephrectomy show a considerably greater increase in the plasma concentration of citric acid than the corresponding group not receiving fluoroacetate (Groups 3 and 4). Parathyroidectomized rats receiving fluoroacetate 4 days later showed a marked increase in

TABLE 1. EFFECT OF FLUOROACETATE UPON THE PLASMA CITRATE AND CALCIUM VALUES OF RATS

Group number*	Description of group	Plasma	
		Citrate, mg.%	Calcium, mg.%
		Av. S.D.	Av. S.D.
1	Normal	3.09 ± .38	11.13 ± .43
2	Normal, injected 5 mg./Kg. sodium fluoroacetate, bled 6 hrs. later	8.03 ± .66	11.95 ± .52
3	Nephrectomized, bled 4 hrs. later	17.96 ± 1.46	15.35 ± 1.19
4	Normal, injected 5 mg./Kg. sodium fluoroacetate, nephrectomized 2 hrs. later, bled 4 hrs. after nephrectomy	25.44 ± 1.67	15.62 ± 1.86
5	Parathyroidectomized, bled 4 days later	2.11 ± .26	7.31 ± .53
6	Parathyroidectomized, injected 5 mg./Kg. sodium fluoroacetate 4 days later, bled 6 hours later	26.50 ± 1.40	15.15 ± 1.41
7	Parathyroidectomized, injected 5 mg./Kg. sodium fluoroacetate 4 days later, nephrectomized 2 hours later, bled 4 hours after nephrectomy	26.30 ± 1.52	15.00 ± 1.48
8	Parathyroidectomized, nephrectomized 4 days later, injected 5 mg./Kg. sodium fluoroacetate after 24 hours, bled 6 hours later	8.90 ± .46	9.70 ± .42
9	Normal, injected 2.5 mg./Kg. sodium fluoroacetate, bled 6 hours later	6.70 ± .54	11.60 ± .48
10	Parathyroidectomized, injected 2.5 mg./Kg. sodium fluoroacetate 4 days later, bled 6 hours later	8.10 ± .55	8.50 ± .44
11	Parathyroidectomized, injected 2.5 mg./Kg. sodium fluoroacetate 4 days later, nephrectomized 2 hours later, bled 4 hours after nephrectomy	19.20 ± 1.10	9.40 ± .26
12	Parathyroidectomized, given parathyroid extract (30 I.U.) on day of operation and twice daily for 3 days	3.07 ± .32	11.20 ± .28
13	Parathyroidectomized, given parathyroid extract (30 I.U.) on day of operation and twice daily for 3 days, injected 5 mg./Kg. sodium fluoroacetate on 4th day, bled 6 hours later	15.60 ± 1.06	12.30 ± .42
14	5000 I.U. Vitamin D daily for 7 days, parathyroidectomized on 4th day, bled on 8th day	4.10 ± .36	12.45 ± .54
15	5000 I.U. Vitamin D daily for 7 days, parathyroidectomized on 4th day, injected 5 mg./Kg. sodium fluoroacetate on 8th day, bled 6 hours later	10.40 ± .96	12.25 ± .38
16	5000 I.U. Vitamin D daily for 7 days, parathyroidectomized on 4th day, injected 5 mg./Kg. sodium fluoroacetate on 8th day, nephrectomized 2 hours later, bled 4 hours after nephrectomy	26.00 ± 2.00	15.25 ± 1.46

* Ten rats to each group.

plasma citrate as compared to the effects of parathyroidectomy alone (Groups 5 and 6). Nephrectomy had no further effect on the plasma calcium and citric acid values (Group 7) of parathyroidectomized animals given 5 mg./Kg. of fluoroacetate. Groups 6 and 7 were the most prone of all the animals to manifest tetany and convulsions following fluoroacetate administration. If the administration of fluororoacetate to the parathyroidectomized rats was delayed until 24 hours after nephrectomy, it had relatively little effect on the plasma calcium and citrate values (Group 8.

TABLE 2. COMPARISON OF PLASMA CALCIUM AND CITRIC ACID VALUES OF FLUOROACETATE TREATED AND CONTROL RATS

Groups compared*	Per cent probability		Groups compared*	Per cent probability	
	Calcium	Citric acid		Calcium	Citric acid
1–2	$<.01$	$<.05$	5–10	$<.01$	$<.01$
1–9	$<.01$	$>.05$	6–7	$>.05$	$>.05$
2–4	$<.01$	$<.01$	6–10	$<.01$	$<.01$
2–6	$<.01$	$<.01$	6–13	$<.01$	$<.01$
2–9	$<.01$	$>.05$	7–8	$<.01$	$<.01$
2–13	$<.01$	$>.05$	7–11	$<.01$	$<.01$
2–15	$<.01$	$>.05$	9–10	$<.01$	$<.01$
3–4	$<.01$	$>.05$	10–11	$<.01$	$<.01$
4–7	$>.05$	$>.05$	12–13	$<.01$	$<.01$
4–16	$>.05$	$>.05$	14–15	$<.01$	$>.05$
5–6	$<.01$	$<.01$	15–16	$<.01$	$<.01$

* See Table 1 for identity of groups.

See also Figures 1 and 2 of preceding paper). The values obtained on Group 8 are in sharp contrast to those obtained on Group 7, yet the only difference in treatment of these two groups was in the time of fluoroacetate injection in relation to nephrectomy.

The increase in plasma calcium and citric acid produced by injecting 2.5 mg./Kg. of fluoroacetate was less than produced by the larger dose (Groups 9, 10 and 11). Use of the smaller dose permitted demonstration of an effect of nephrectomy on the citric acid response of parathyroidectomized animals (Groups 10 and 11. Compare with Groups 6 and 7 and Fig. 2 of previous paper.) Parathyroidectomized rats given parathyroid extract manifested a calcium and citric acid response to fluoroacetate injection more nearly resembling the response of the normal animal (Groups 13 and 2). The same may be said of parathyroidectomized rats given sufficient Vitamin D to restore the plasma calcium value to normal prior to fluoroacetate administration (Group 15). Nephrectomy of the Vitamin D treated parathyroidectomized rats given fluoroacetate resulted in a marked rise in plasma citrate and calcium similar to that observed in normal animals.

DISCUSSION

Blockade of citric acid destruction in the body by fluoroacetate must be incomplete or else the animal will die in convulsion.[3] Incompleteness of

[3] A few hypocalcemic animals given 5 mg./Kg. of fluoroacetate did die in convulsions.

the blockade is also evidenced by the marked response of certain fluoroacetate injected groups to nephrectomy (Groups 4, 11 and 16). It seems probable that blockade of the citric acid destroying mechanisms may be more readily effected in some tissues than in others. That blockade of the mechanism concerned with the removal of circulating citrates must have been close to maximal in certain groups is suggested by their uniformity of response (Groups 4, 6, 7 and 16) and by the fact that nephrectomy failed to produce any further elevation of the plasma values in Group 7 as compared to Group 6.

It is evident from a review of the data that the concentration of calcium in the body fluids, hence in the tissues as well, must have a marked influence on the turnover of citrate that occurs or in the effectiveness of fluoroacetate in blocking the destruction of citric acid as judged by plasma concentration of citrate (Groups 6, 13 and 15). Undoubtedly, the intracellular accumulation of citrate must lead to calcium citrate formation and thereby promote greater neuromuscular irritability and perhaps further citrate formation. Reduction in intracellular calcium ion concentration may alter cell permeability and permit more citrate to escape from the blockaded tissue.

The marked increase in the plasma concentration of citric acid in fluoroacetate injected hypoparathyroid animals demonstrates that a considerable turnover of citrate occurs between plasma and tissues in the absence of the parathyroid glands. The "maximal" response achieved in certain groups after nephrectomy (Groups 4 and 16) suggests that the renal mechanism may be less readily blockaded than the tissue mechanism concerned with citrate removal from the circulation. The extent to which the kidney of the hypoparathyroidectomized animal is metabolizing or excreting citrate is suggested by the response of the partially blockaded hypoparathyroid animal to nephrectomy (Group 10). That the extra renal mechanism of plasma citrate removal is largely blockaded by the 5 mg. dose of fluoroacetate is suggested by the similarity of response of the normal fluoroacetate poisoned group to nephrectomy (Group 4) with that of Groups 6 and 7.

The failure of fluoroacetate injection 24 hours after nephrectomy to produce any marked effect on the concentration of circulating citrates could be due to a reduced turnover of circulating citrates or to reduced effectiveness of the blocking agent. It seems unlikely that the effectiveness of the blocking agent would be reduced, particularly in the hypocalcemic animal so that a reduced turnover of citrates appears to be the most probable explanation. Another possibility is that the liver, which fluoroacetate does not blockade (4), metabolizes circulating citrates to a greater extent 24 hours after nephrectomy.

The extent to which the liver may be metabolizing citrates under the conditions existing in these experiments has not been established. Potter

and Busch (4) (1950) demonstrated that no accumulation of citrates occurred in this organ in the presence of fluoroacetate concentrations that blocked citrate destruction in other tissues. Martensson (6) perfused isolated liver with citrate-rich blood, but found no evidence of its removal under these circumstances. Boothby and Adams (7) reported an increase in urinary excretion of citric acid some hours after hepatectomy in the dog and Thunberg (8) found that the plasma citric acid increase to oral citrate was greater in patients with liver disease than in normal subjects. Comparison of the citric acid content of portal and hepatic vein blood has failed to demonstrate any difference in a few observations made on normal fasting dogs.

It also seems probable that a limited supply of readily mobilizable citrate exists somewhere in the body. The fundamental questions as to whether or not the presence of the parathyroid gland directly or indirectly influences the mobilization or metabolism of citric acid remains unanswered. That the calcium concentration influences either citric acid turnover or mobilization is suggested by the influence of plasma calcium concentration on the response to fluoroacetate administration. It is hoped that *in vitro* studies currently in progress may help to clarify the relation of calcium concentration to the oxidation of citric acid.

The relatively stable concentrations of citrate normally present in plasma indicate that the animal organism must possess a specific mechanism for regulating the plasma concentration of this substance. The continuous turnover of plasma citric acid must contribute to homeostasis in some manner. Leakage of citric acid from the muscles or kidney would hardly suffice as a stable source of plasma citrate. Some relation between plasma calcium and citric acid is evident since hypocalcemia is accompanied by a slight decline in the citrate concentration and hypercalcemia of sufficient duration produced by parathyroid extract or Vitamin D is accompanied by an increase in the concentration of plasma citrate (1). The two substances may serve to mutually stabilize the concentrations of one another within a given range. Higher concentrations of calcium tending to reduce citrate oxidation by complex formation while increasing citrate concentration would tend to combine with calcium and thereby reduce the calcium ion concentration and lead to further dissociation of the calcium salts in bone. Parathyroid hormone may influence citric acid metabolism only by virtue of its role in regulating calcium ion concentration in body fluids. However, Dixon and Perkins (9) have shown that bone possesses the enzyme mechanisms necessary for the production of high local concentration of citric acid in bone. The effectiveness of hypercitricemia, per se, in promoting calcium mobilization is strikingly demonstrated in the hypoparathyroid rats in which citric acid destruction is reduced by fluoroacetate. These experimental results provide evidence that calcium mobilization by

endogenous citric acid does not require the presence of the parathyroid glands.

SUMMARY

The elevation of plasma citrate produced by sodium fluoroacetate injection into rats was augmented by parathyroidectomy and nephrectomy.

Parathyroid extract and Vitamin D in sufficient amounts to restore the plasma calcium to normal, reduced the response of parathyroidectomized rats to sodium fluoroacetate.

A maximal hypercalcemia of approximately 15 mg.% occurs in rats with elevation of the plasma citrate level.

Endogenous citric acid can mobilize calcium from the skeleton in the absence of the parathyroid glands.

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RELATIVE EFFECT OF VITAMIN D AND PARATHYROID EXTRACT ON PLASMA CALCIUM AND CITRIC ACID OF NORMAL AND THYROPARATHYROIDECTOMIZED DOGS[1]

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HYPERCALCEMIA resulting from calcium mobilization may be accompanied by some elevation of the plasma concentration of citrate. Hypocalcemia is usually accompanied by a reduced plasma citrate value in the parathyroideetomized animal (1). Hypercalcemia produced by calcium chloride infusion for a short time (6 hours) did not elevate the citrate concentration (2). Hypercitricemia produced by the infusion of sodium citrate, nephrectomy or fluoroacetate administration is accompanied by an elevation of plasma concentration of calcium. There appears to be a relation between the serum concentration of calcium and citric acid. It is not apparent whether the degree of hypercitricemia is independent of the amount or mechanism of an endogenous hypercalcemia. It seems important to establish this point in determining whether or not the relation of calcium and citric acid is entirely dependent upon chemical affinity or is related in some other sense depending upon bodily mechanisms. To obtain information bearing on this question, a comparison was made of the relative increase in serum calcium and citric acid in normal and thyroparathyroidectomized dogs made hypercalcemic to a comparable degree, both as regards magnitude and duration of the hypercalcemia.

Vitamin D and parathyroid extract were used to produce the hypercalcemeas. It has been shown by Alwall (3) that the latter will increase the citric acid of plasma, while previous work here (1) showed that the hypercalcemia produced by Vitamin D is accompanied by an increase in the serum concentration of citric acid.

EXPERIMENTAL METHODS AND RESULTS

Seven healthy adult male mongrel dogs were used for this study. The animals were dewormed and maintained on a stock diet of constant composition for 4 to 6 weeks before control values were established. Thereafter, 3 dogs received 150,000 I.U. of Vitamin

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D daily (Drisdol) and 3 animals received 250 I.U. of parathyroid extract[3] (Parathormone, Lilly) subcutaneously daily in divided doses, half in the morning and half in the afternoon. Serum calcium and citric acid values were determined at frequent intervals. When the serum calcium values reached 15 mg.%, Vitamin D was discontinued as the decline in serum calcium was quite gradual. The administration of parthyroid hormone was continued or decreased for 3 days as required to give a curve resembling the hypercalcemia produced by Vitamin D. Following the return of all serum calcium values to normal, all 6 dogs were thyroparathyroidectomized and allowed to develop tetany. One of the animals died and was replaced. After hypocalcemia became marked, the animals were again given the hypercalcemic agents as previously noted and the serum values for calcium and citric acid were followed. The laboratory and surgical procedures have been described previously (1).

TABLE 1. RELATIVE EFFECT OF VITAMIN D AND PARATHORMONE ON PLASMA CALCIUM AND CITRIC ACID VALUES OF DOGS*

Experimental condition			Plasma values mg. %	
State	Comment	Duration, days	Calcium	Citrate
Normal	Control		11.15 ± .36	4.10 ± .30
	Vit. D	4–13	13.37 ± .40	7.33 ± .79
	Vit. D	7–17	15.63 ± .18	10.98 ± .68
	Vit. D Discon'td	6–12	13.58 ± .11	8.03 ± .67
	Vit. D Discon'td	23–30	11.08 ± .93	4.48 ± .23
Normal	Control		10.30 ± .35	3.90 ± .20
	Pth.	5– 9	13.53 ± .19	6.83 ± .55
	Pth.	7–11	15.20 ± .10	7.70 ± .63
	Pth. Discon'td	2– 4	13.03 ± .54	6.60 ± .72
	Pth. Discon'td	14–18	10.63 ± .30	4.13 ± .24
Pth'omy	Control		5.97 ± .34	2.75 ± .48
	Vit. D	5–10	10.82 ± .86	5.87 ± 1.51
	Vit. D	13–14	13.70 ± .13	7.93 ± 1.12
	Vit. D	15–17	15.45 ± .14	10.73 ± .31
	Vit. D Discon'td	14–30	11.92 ± .41	5.80 ± .54
	Vit. D Discon'td	38–57	9.64 ± .36	3.40 ± .37
Pth'omy	Control		6.27 ± .21	2.42 ± .38
	Pth.	3– 4	10.85 ± .25	4.55 ± .58
	Pth.	4– 6	12.95 ± .55	5.62 ± .39
	Pth.	6– 8	15.58 ± .28	7.48 ± .42
	Pth. Discon'td	3– 4	13.53 ± .34	5.90 ± .46
	Pth. Discon'td	5– 6	10.08 ± .37	4.10 ± .15
	Pth. Discon'td	9–11	7.04 ± .43	2.80 ± .30

* Three dogs in each group.
Vit. D = Vitamin D
Discon'td. = Discontinued
Pth. = Parathyroid Hormone
Pth'omy = Thyroparathyroidectomy

Pertinent data regarding the response of various animals to the hypercalcemic agents are included in Tables 1 and 2. It will be seen that the time required to produce an hypercalcemia of 15 mg.% varied from 7 to 17 days and took somewhat longer with Vitamin D than with the parathyroid extract. The normal ratio of calcium to citric acid in plasma expressed

[3] The authors wish to acknowledge their indebtedness to Dr. G. W. Probst of Eli Lilly and Company for a generous supply of parathyroid extract.

TABLE 2. PLASMA CALCIUM AND CITRIC ACID CHANGES IN NORMAL AND THYROPARATHYROIDECTOMIZED DOGS GIVEN VITAMIN D OR PARATHYROID EXTRACT

Legend	Normal dogs given 150,000 units vitamin D daily			Normal dogs injected 250 units parathyroid extract daily			
	#201	#236	#237	#258	#271	#275	#276
Normal ratio of Ca./Cit.	3.10	2.59	2.55	2.59	2.16	2.89	2.46
No. days for Serum Ca. to reach 15 mg.%	7	17	12	11		7	7
Ratio Ca./Cit. at maximum hypercalcemia	1.40	1.53	1.44	1.80		2.00	2.11
Ratio of increase Ca./Cit. above normal in hypercalcemic state	0.61	0.76	0.79	1.03		1.21	1.22
	Thyroparathyroidectomized dogs						
No. days for Serum Ca. to reach 15 mg.%	17	17	15	8	7		6
Ratio Ca./Cit. at maximum hypercalcemia	1.40	1.45	1.45	2.01	2.03		2.09
Ratio of increase Ca./Cit. above normal in hypercalcemic state	0.63	0.67	0.65	1.36	1.61		1.38

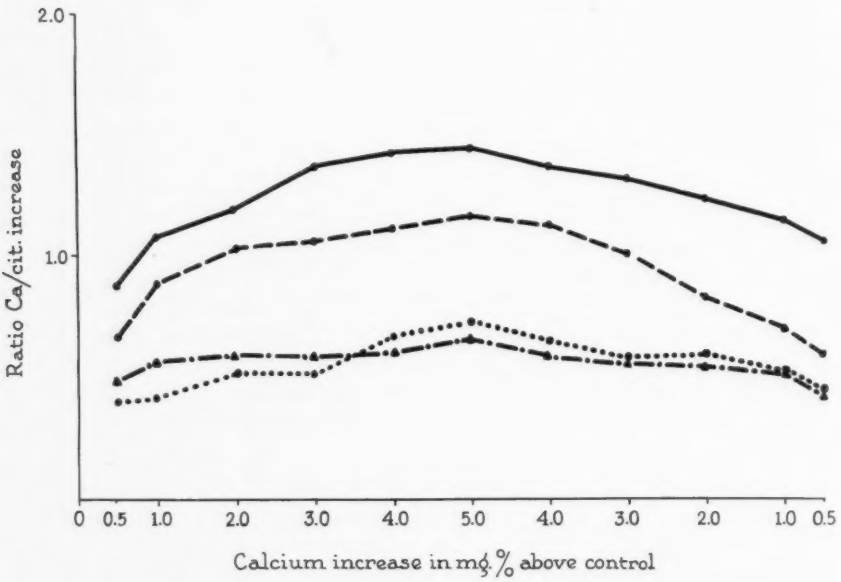

FIG. 1.

Solid line (——) Parathyroidectomized dogs receiving parathyroid extract.
Broken line (– – –) Normal dogs receiving parathyroid extract.
Broken and dotted line (·—·—) Normal dogs receiving Vitamin D.
Dotted line (· · · ·) Parathyroidectomized dogs receiving Vitamin D.

Showing the ratio of change of calcium to citric acid in relation to varying degrees of hypercalcemia in normal and thyroparathyroidectomized dogs made hypercalcemic by Vitamin D or parathyroid extract. Calcium and citric acid expressed as mg.%. Each curve is the average of serial values obtained on 3 animals.

on a weight basis was usually between 2 and 3. The ratio decreased in the hypercalcemic animals and was different for the two hypercalcemic agents. Vitamin D produced a relatively greater increase in citric acid in relation to a given calcium elevation than did parathyroid extract. The ratio of the increase in serum calcium to increase in citrate concentration at the peak of the hypercalcemia is shown in Table 2. The ratios are all lower for the Vitamin D animals, both before and after parathyroidectomy. There is no overlap in the ratio of the increase in calcium and citric acid produced by the two substances. The change in the ratio of the increase in relation to varying degrees of hypercalcemia are shown in Figure 1. The difference in the relative effect of Vitamin D and of parathyroid extract on calcium and citric acid is clearly shown in this figure. The increase in citrate was relatively greater in the Vitamin D treated animals than in those receiving parathyroid extract. This was true both during the development of the hypercalcemia and while it was subsiding. The effect of Vitamin D was not influenced by the parathyroid gland since the ratio of the increase was practically identical before and after parathyroidectomy. However, the thyroparathyroidectomized dogs receiving parathyroid extract had relatively less rise in citric acid than prior to surgery. This difference between the response of the normal and thyroparathyroidectomized animal is apparent throughout the entire range of hypercalcemia, both during the phase of calcium increase and decline.

DISCUSSION

The hypercitricemia produced by parathyroid hormone was significantly less (approximately one-half) that produced by Vitamin D for a corresponding hypercalcemia. The ratio of the increase of calcium and citrate produced by Vitamin D is essentially the same regardless of the magnitude of the change as indicated by the slope of its curve in Figure 1. The relatively greater increase in calcium than of citric acid produced by parathyroid extract indicates that the relative amounts of calcium to citric acid in plasma is not fixed and cannot be entirely explained on a basis of mutual stabilization due to complex formation. It is also evident from inspection of the curves in Figure 1, that the effect of parathyroid extract on the ratio of calcium to citrate increase becomes apparent as soon as any hypercalcemia develops, while there is considerable lag before the calcium rise produced by Vitamin D is associated with a slight tendency toward a change in ratio. Unpublished experiments from this laboratory have shown that the acute hypercalcemia resulting from injection of a single large dose of parathyroid extract into dogs may not be accompanied by any change in the plasma concentration of citric acid.

The studies by Steenbock and Bellin (4, 5) have shown that the citric acid content of bone and soft tissues of young and old rats, as well as plasma and urine is influenced by physiological amounts of Vitamin D.

Harrison (6, 7) (1952, 1954) found that Vitamin D raised the citric acid content of the serum and urine of rachitic infants.

The mechanism of the hypercalcemic effect of both substances is unknown. Presumably, the hypercalcemic effect of parathyroid hormone is largely due to its action on bone, while that of Vitamin D is related both to its effect in promoting calcium absorption, as well as to a direct decalcifying effect on bone. Dietary calcium would contribute to some extent to the hypercalcemia produced by both agents, but would probably be a greater contributing factor to the Vitamin D hypercalcemia. It seems unlikely that the dietary calcium has any bearing on the observed differences in the effect of Vitamin D and parathyroid extract. When the hypercalcemia reaches approximately 15 mg.%, most dogs largely stopped eating so that at least a part of the results on all animals were associated with anorexia and the hypercalcemia at this time must have been endogenous in origin.

SUMMARY

A fasting serum calcium value of approximately 15 mg.% produced in normal or thyroparathyroidectomized dogs by Vitamin D is usually associated with a plasma citric acid value of 10–11 mg., while a comparable hypercalcemia produced by parathyroid extract is accompanied by a maximum plasma citrate value of 7.5–8.0 mg.%. The ratio of calcium to citric acid increase was always greater when parathyroid extract was given than when Vitamin D was administered. The relative increase in calcium to citric acid was greatest in thyroparathyroidectomized animals given parathyroid extract.

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THE INFLUENCE OF AGE ON THE DEVELOPMENT OF ADRENAL-REGENERATION HYPERTENSION*

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RECENT experiments (1, 2) have shown that uninephrectomized rats given 1% sodium chloride to drink develop hypertension and widespread vascular disease following adrenal-enucleation. The rise in blood pressure occurs as the adrenal cortex regenerates from the capsule and cortical cells adherent to the capsule (3–7) which remain in situ following the operative procedure, and, hence, has been designated "adrenal-regeneration hypertension." The initial observations were made on young rats, and since Timiras, Faribault and Selye (8) have shown that the hypertensive and vasculotoxic actions of desoxycorticosterone acetate were particularly severe in young animals, the present experiment was performed to study the influence of age on the development of adrenal-regeneration hypertension and the accompanying morphologic lesions.

MATERIALS AND METHODS

Female Sprague-Dawley rats were used. Twenty immature rats weighing 50–59 grams and 20 young mature rats weighing 218–244 grams were uninephrectomized and given 1% sodium chloride to drink *ad libitum* and a diet of Purina Laboratory Chow. Ten of the immature rats (Group I) and 10 of the young mature rats (Group III) served as controls. The remaining 10 immature rats (Group II) and 10 young mature rats (Group IV) had the right adrenal removed and the left adrenal enucleated according to the method of Ingle and Higgins (3) in which as much as possible of the glandular tissue was extruded through a capsular incision by the gentle application of pressure with curved forceps.

Body weight and systolic blood pressure, the latter by the microphonic manometer method of Friedman and Freed (9), were determined at the beginning of the experiment and weekly thereafter. Consumption of 1% sodium chloride was calculated for each rat at weekly intervals and expressed for each group as the average 24 hour intake per rat. At the end of 8 weeks blood was withdrawn from the abdominal aorta with the rat under ether anesthesia. The levels of serum sodium and potassium were determined on the Beckman DU flame photometer, and serum chloride was measured by the method of Schales and Schales (10). Kidney, heart, brain, adrenal(s), liver, spleen, thymus,and pituitary were removed immediately after death and weighed fresh on a gramatic balance. Portions of the pancreas, mesentery and gastro-intestinal tract were also taken for microscopic study. All tissues were fixed in Zenker-formal and stained with hematoxylin and eosin. One adrenal from the control rats and one half of the regenerated adrenal

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from the rats of Groups II and IV were fixed in 10% formalin. Sections of the adrenals were cut at 8 microns on the freezing microtome and stained with sudan IV using a hematoxylin counter stain.

RESULTS

Two rats in Group II died during the experiment and the data accumulated on these rats was discarded. In addition it was necessary to kill 1 rat from Group II after 6 weeks and 1 after 7 weeks because of their poor condition; control rats from Group I were killed at the same time as these latter animals. The data from these rats proved statistically indistinguishable from those animals in their respective groups which survived the full 8 weeks, and, hence, the data from each complete group was considered together.

The average body weights, saline consumption and systolic blood pressure of the four groups are illustrated in Figure 1. The difference in the growth rate between the immature and the young mature rats is readily apparent. The average increase in the body weight of the 2 groups of immature rats during the experiment was 155 and 146 gm., a weight gain of almost 300%, while in the 2 groups of young mature rats the gain in body weight was only 36 and 48 gm., an increase of approximately 20%. There was no significant difference between the daily saline consumption of either the immature adrenal-enucleated rats (Group II) and their controls (Group I), or between the young mature adrenal-enucleated rats (Group IV) and their controls (Group III). However, it is noteworthy that for the first 3 weeks of the experiment the daily saline intake of both groups of immature rats was significantly greater ($P < 0.05$) than it was in the young mature rats, but that thereafter a significant difference ($P < 0.05$) existed only between the immature and young mature adrenal-enucleated animals.

Despite the absence of polydipsia in the adrenal-enucleated rats of both ages as compared to their respective controls, a highly significant ($P < 0.01$) elevation of blood pressure occurred in the immature adrenal-enucleated rats, while in the young mature adrenal-enucleated rats only a modest elevation, statistically not significant, developed. Furthermore, the systolic blood pressure in the immature adrenal-enucleated rats was significantly higher ($P < 0.01$) than in the similarly treated young mature animals.

The average organ weights and serum electrolyte levels for the 4 groups are shown in Table 1. There was significant ($P < 0.01$) cardiac and renal hypertrophy in the immature adrenal-enucleated rats (Group II) while no hypertrophy of these organs occurred in the young mature adrenal-enucleated rats (Group IV). The weight of the regenerated adrenal cortex in the immature rats (Group II) was not significantly different ($P < 0.8$) from the combined weight of both adrenal glands in their controls (Group I), while in the young mature rats (Group IV) the weight of the regenerated adrenal was significantly less ($P < 0.01$) than the weight of both adrenals in their control rats (Group III). The liver and spleen were also significantly

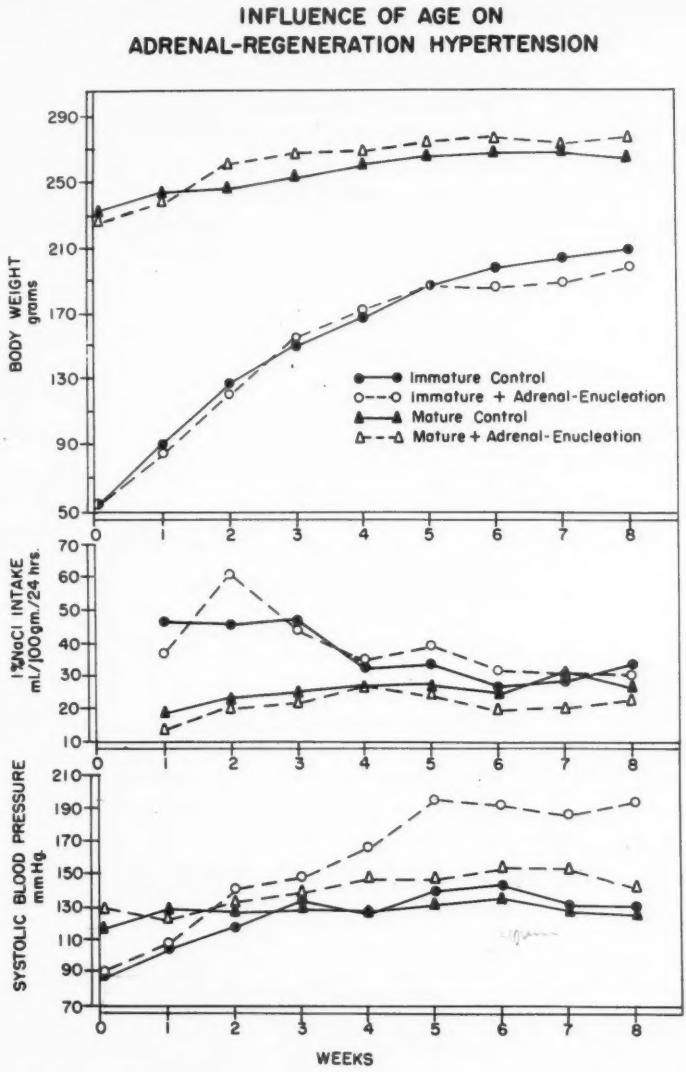

FIG. 1. Body weight, saline consumption and systolic blood pressure changes in immature and young mature uninephrectomized salt-treated rats following adrenal-enucleation.

enlarged ($P < 0.01$ and $P < 0.05$) in the immature adrenal-enucleated rats (Group II) but not in the young mature adrenal-enucleated rats (Group IV). No change occurred in the size of the thymus, brain or pituitary gland in either group of adrenal-enucleated rats as compared to their respective controls.

Electrolyte studies showed (Table 1) that a small but statistically significant ($P < 0.01$) elevation of serum sodium occurred in the immature adrenal-enucleated rats as compared to the immature controls. This is at variance with previously published results in which no such difference was observed, but evaluation of the real significance of the present observation should take into consideration the fact that the young mature rats of both Groups III and IV had higher serum sodium levels than the immature

TABLE 1. ORGAN WEIGHTS AND SERUM ELECTROLYTE VALUES OF IMMATURE AND YOUNG MATURE UNINEPHRECTOMIZED, SALT-TREATED ADRENAL-ENUCLEATED RATS AND THEIR CONTROLS

Group and treatment	No. of rats	Organ weights, mg. per 100 gm. body weight*								Serum electrolytes, mEq./L.*		
		Kidney	Heart	Brain	Adrenal(s)	Liver	Spleen	Thymus	Pituitary	Na	K	Cl
I Immature control	8	603 ±19	358 ±15	1146 ±30	27.7 ±0.8	3270 ±171	219 ±11	118 ±12	6.6 ±0.24	143.0 ±1.1†	4.5 ±0.1†	110.0 ±0.8†
II Immature adrenal-enucleated	8	890 ±74	534 ±42	1344 ±114	27.0 ±2.7	5192 ±433	350 ±61	101 ±14	6.6 ±0.1	148.6 ±1.43‡	4.6 ±0.1‡	110.0 ±1.7‡
III Mature control	10	438 ±12	318 ±7	955 ±24	24.2 ±1.1	2926 ±52	186 ±9	81 ±7	7.0 ±0.24	149.6 ±0.8	4.4 ±0.4	107.8 ±1.2
IV Mature adrenal-enucleated	10	464 ±16	328 ±6	912 ±14	18.1 ±0.9	2921 ±69	193 ±9	71 ±4	6.6 ±0.22	150.9 ±1.1	4.5 ±0.24	110.1 ±1.8

* All values accompanied by the standard error of the mean.
† Determinations on 6 rats only.
‡ Determinations on 5 rats only.

adrenal-enucleated animals yet did not develop either hypertension or vascular changes.

Microscopic Studies.—These observations are summarized in Table 2 from which it is apparent that the morphologic changes were much more severe in the immature adrenal-enucleated rats than they were in the young mature adrenal-enucleated animals, where, indeed, lesions were virtually absent. No morphologic changes were present in the control rats of either age.

Since a detailed account of the morphologic changes which accompany this type of experimental hypertension has been published elsewhere (11), a brief description of these lesions will suffice here. The principal vascular lesion was fibrinoid necrosis of arterioles in the kidney (Figs. 2 and 3), heart (Fig. 5), adrenal capsule, thymus, pancreas, brain (Fig. 6), mesentery and intestinal wall (Fig. 7), and is virtually identical to that found in malignant hypertension in the human being. Larger arteries of the pancreas and mesentery frequently showed the medial necrosis, endothelial hyperplasia, subendothelial PAS-positive material and mural inflammatory reaction characteristic of periarteritis nodosa in this species (Fig. 8). The morphologic changes in the kidney also included glomerulonecrosis, dilatation of tubules, slight thickening of tubular basement membranes and focal

basophilia and hyperplasia of epithelial cells lining the tubules (Fig. 2 and 3). There were foci of necrosis and hemorrhage in the regenerated adrenals, and the brains of these rats frequently showed edema, focal hemorrhage, neuronal degeneration and old cystic infarcts (Fig. 6). These changes appeared to result from occlusion of arterioles secondary to fibrinoid necrosis of their walls.

The adrenal cortex of both immature and young mature control rats was

TABLE 2. SUMMARY OF MORPHOLOGIC FINDINGS IN IMMATURE AND YOUNG MATURE UNINEPHRECTOMIZED, SALT-TREATED ADRENAL-ENUCLEATED RATS AND THEIR CONTROLS

Organ	Group and treatment			
	I Immature control	II Immature adrenal-enucleated	III Young mature control	IV Young mature adrenal-enucleated
Kidney	Slight dilatation of tubules with focal basophilia of tubular epithelium	Glomerulonecrosis and fibrinoid necrosis of arterioles; thickening of basement membrane of tubules; dilatation and hyaline casts in tubules. Lesions present in all rats varying from slight to severe	No lesions	Slight necrosis of a few glomeruli; no fibrinoid necrosis of arterioles; no tubular changes
Heart	No lesions	Fibrinoid necrosis of coronary arterioles. Lesions present in 4 out of 8 rats	No lesions	No lesions
Brain	No lesions	Fibrinoid necrosis of arterioles, edema, neuronal degeneration, focal hemorrhage, and old and recent infarcts. Lesions present in 5 out of 8 rats	No lesions	No lesions
Adrenal	Wide zona glomerulosa containing almost no lipoid; lipoid of zona reticularis and inner zona fasciculata, slightly less than seen in untreated rats	Thin zona glomerulosa containing as much lipoid as zona reticularis and zona fasciculata; lipoid present in regenerated cortex as coarse globules. Changes essentially the same in all rats	Zona glomerulosa wider and with more lipoid than in Group I	Zona glomerulosa slightly wider than in Group II but still contains much lipoid
Thymus	No lesions	Fibrinoid necrosis of arterioles in connective tissue. Lesions present in 1 out of 8 rats. Thymic tissue normal	No lesions	No lesions
Spleen	No lesions	Fibrinoid necrosis of arterioles. Lesions present in 1 out of 8 rats. Lymphoid tissue normal	No lesions	No lesions
Pancreas, mesentery and intestine	No lesions	Fibrinoid necrosis of arterioles and periarteritis nodosa of arteries. Lesions present in 5 out of 8 rats	No lesions	No lesions

normal in appearance in the hematoxylin and eosin stain. However, in agreement with previously reported observations (11), the zona glomerulosa of the adrenals from the immature control rats was virtually devoid of sudanophilic material (Fig. 9) while a considerable amount of lipoid appeared in this zone of the adrenals from the mature control animals (Fig. 11). The zona reticularis and inner zona fasciculata of the adrenals from control rats of both ages showed slightly less lipoid than is usually seen in these zones of adrenals from normal untreated rats of the same age and strain. In contrast to the appearance of the adrenal cortex of the control rats, the zona glomerulosa of the regenerated adrenals from both immature and young mature rats appeared narrow and was composed of small cells

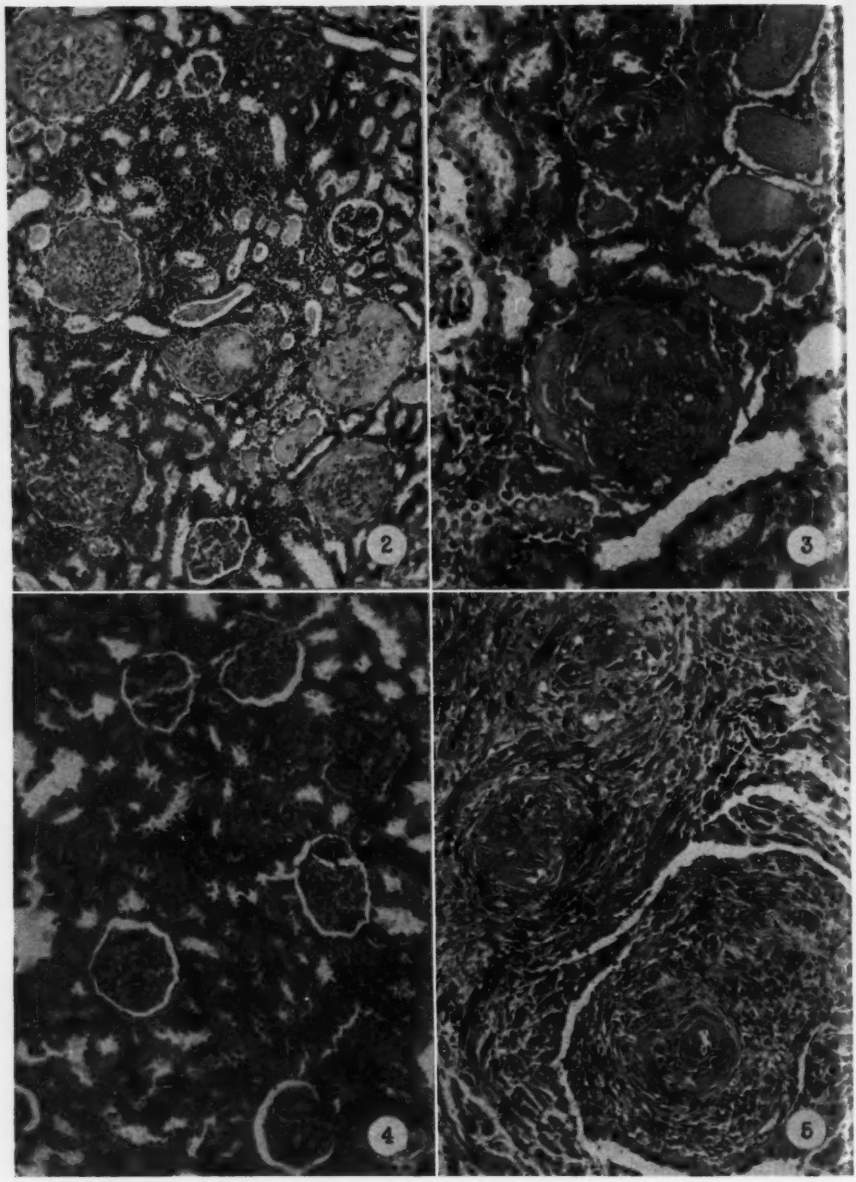

PLATE 1

FIG. 2. Kidney of immature, adrenal-enucleated rat showing extensive nature of glomerular sclerosis and necrosis. Hematoxylin and eosin. ×72.

FIG. 3. Higher magnification of same kidney as in Fig. 2 illustrating glomerulonecrosis, arteriolonecrosis, tubular dilatation and hyaline casts. Hematoxylin and eosin. ×228.

FIG. 4. Kidney of young mature, adrenal-enucleated rat showing complete absence of lesions. Hematoxylin and eosin. ×100.

FIG. 5. Heart of immature, adrenal-enucleated rat containing numerous arterioles undergoing necrosis with extravasation of hyaline material and perivascular fibroblastic proliferation. Hematoxylin and eosin. ×144.

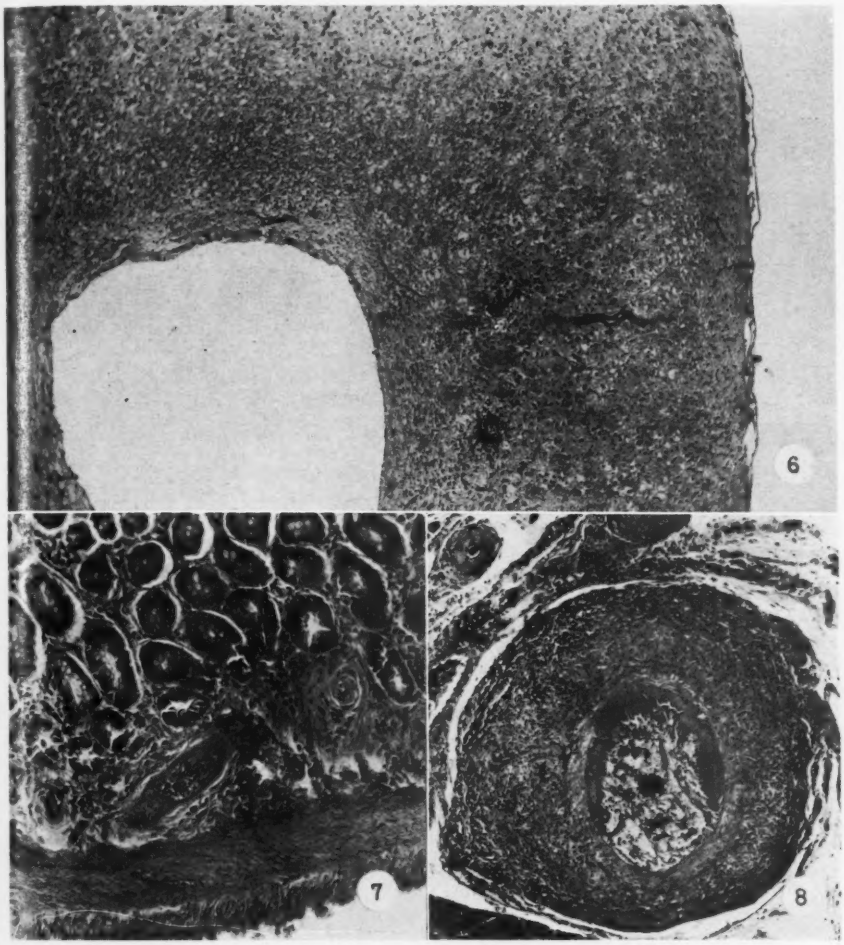

PLATE 2

FIG. 6. Brain of immature, adrenal-enucleated rat showing edema, neuronal degeneration, arteriolar necrosis, focal hemorrhage and old cystic infarct. Hematoxylin and eosin. ×62.

FIG. 7. Intestine from immature, adrenal-enucleated rat containing arterioles undergoing sclerosis and necrosis. Hematoxylin and eosin. ×144.

FIG. 8. Pancreatic artery from immature, adrenal-enucleated rat showing typical changes of periarteritis nodosa in this species. Hematoxylin and eosin. ×72.

with dark nuclei and scanty cytoplasm while the cells of the zona fasciculata and zona reticularis were large with small nuclei and much clear, foamy cytoplasm. The regenerated cortices also differed from the control glands in that there was as much or more lipoid in the zona glomerulosa as in the other two zones (Figs. 10 and 12).

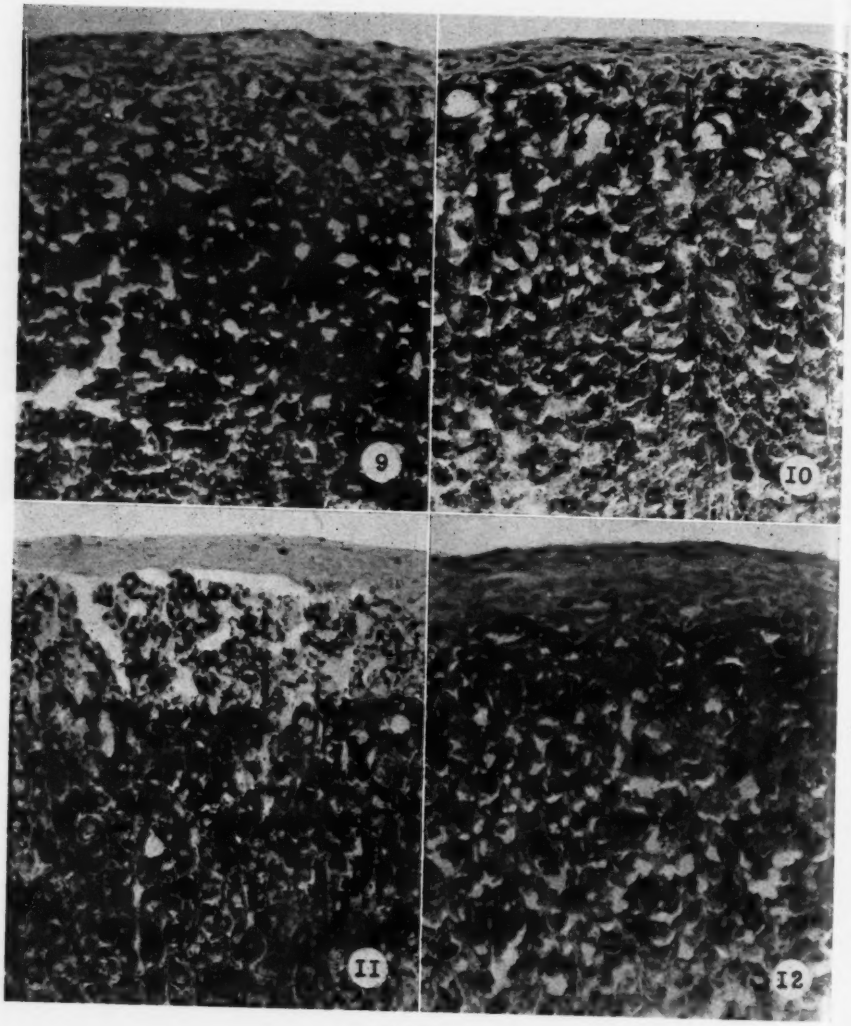

PLATE 3

FIG. 9. Adrenal cortex of immature control rat showing wide zona glomerulosa containing little lipoid. Sudan IV and hematoxylin. ×228.

FIG. 10. Regenerated adrenal cortex of immature rat showing narrow zona glomerulosa which contains much lipoid. Sudan IV and hematoxylin. ×228.

FIG. 11. Adrenal cortex of young mature control rat showing wide zona glomerulosa containing more lipoid than in immature control rat. Sudan IV and hematoxylin. ×228.

FIG. 12. Regenerated adrenal cortex of young mature rat in which zona glomerulosa is as wide as in regenerated cortex of immature rat (Fig. 10) and in which much lipoid is present. Sudan IV and hematoxylin. ×228.

DISCUSSION

These experimental results confirm the observations reported previously (1, 2) that uninephrectomized, salt-treated, immature Sprague-Dawley rats develop hypertension and cardiovascular-renal lesions following unilateral adrenalectomy and contralateral adrenal-enucleation. Studies on the rate of regeneration of the adrenal cortex (3) have indicated that maximum adrenal size is attained 4-6 weeks after surgical enucleation. Therefore, it appears that in the present experiment the blood pressure began to rise during regeneration of the cortex and reached the highest level coincident with maximum regeneration. Since the only difference between the adrenal-enucleated and the control rats in this and previous experiments has been the presence of a regenerating adrenal gland in the former animals, it is concluded that the development of hypertension and cardiovascular-renal lesions is related to the functional activity of the regenerating cortical tissue.

The nature of the secretory product of the regenerating adrenal cortex and the mechanism of its participation in the genesis of the hypertension and vascular lesions is at present obscure. However, it should be recalled that evidence has been presented (6, 12, 13) that regenerating cortical tissue secretes increased quantities of glucocorticoids, and the observations that rats bearing regenerating adrenals have normal serum electrolyte levels (14), do not require extra sodium chloride for survival (6) and prefer water to saline if allowed self-selection (15) attest to the ability of these glands to secrete mineralocorticoids also. While both glucocorticoids such as cortisone (16) and mineralocorticoids such as desoxycorticosterone acetate (17) have been shown to produce hypertension, the absence of thymic atrophy and the simultaneous presence of hypertrophy of the spleen and liver, slightly elevated serum sodium, and periarteritis nodosa in immature rats bearing regenerated adrenals suggests that the secretory aberration of these glands involves an increased secretion of steroids which exert mineralocorticoid activity rather than glucocorticoid activity. If this is so, then it might be expected that the corticoids secreted in excess were either corticosterone, aldosterone, or both since Singer and Stack-Dunne (18) have shown that these are the principal steroids secreted by the adrenal cortex of the rat. Additional experiments are planned to investigate this aspect of the problem.

In contrast to the severe hypertension and vascular lesions which developed in the immature adrenal-enucleated rats, the similarly treated young mature animals showed only a modest elevation of blood pressure and virtual absence of any vascular changes. These divergent result in rats of different ages are difficult to explain but several observations are, perhaps, pertinent. The weight of the regenerated adrenal in the mature rats was significantly less than the weight of the regenerated gland in the

immature rats when expressed as mg. per 100 gm. of body weight, although they were the same weight in both age groups when expressed in mg. In vitro experiments of Birmingham, Debarats and Hoffman (19) have shown that adrenal glands from young rats (less than 2 months-of-age), secrete less total steroids than do adrenals from older rats (4 to 8 months-of-age and over 24 months-of-age), but that the steroid output is nearly twice as high in the adrenals from young rats as in the glands from older animals when calculated per unit of body weight. It is conceivable, therefore, that hypertension and vascular lesions developed in the immature but not in the mature rats of the present experiment because of a greater secretory capacity of the regenerated adrenal cortex relative to body weight in the young animals.

Any attempted explanation of the differences between the immature and young mature adrenal-enucleated rats cannot ignore the observation that the saline consumption of the former animals was approximately twice that of the latter animals when expressed in ml. per 100 gm. of body weight. Thus the lower saline intake per unit of body weight which was observed in both groups of young mature rats may have been sufficient to protect those with regenerating adrenal-cortical tissue from developing hypertension and vascular lesions. This possibility is based, of course, on the assumption that sodium chloride plays a role in the genesis of this form of experimental hypertensive vascular disease such as has been demonstrated for the experimental hypertensive vascular disease produced by desoxycorticosterone acetate in this species (20-22). That such is the case has been indicated by recent experiments (23) in which immature adrenal-enucleated rats given water instead of 1% sodium chloride solution to drink failed to develop either significant hypertension or vascular lesions. However, it seems unlikely that increased saline consumption alone can explain the development of hypertension and vascular lesions in immature adrenal-enucleated rats since in this and previous experiments (1, 2) control animals with the same intake of saline did not become hypertensive and did not show vascular changes. These observations suggest, therefore, that excess available sodium chloride is necessary for the development of hypertensive vascular disease during adrenal cortical regeneration but that some factor or factors are more important than saline consumption alone.

Another difference between the immature and young mature rats was their rate of body growth. Inasmuch as the immature rats were undergoing their most rapid growth during the course of the experiment it may be assumed that there was either a greater production of growth hormone in these rats than in the young mature animals or that the tissues of these rats are more responsive to growth hormone than are the tissues of the older animals. Significantly, in this regard Selye (24) has shown that administration of growth hormone to uninephrectomized rats allowed 1% sodium chloride to drink produced hypertension and vascular lesions simi-

lar to those seen in the immature adrenal-enucleated rats of the present experiment. Since Selye's observations were made in rats with an intact anterior-hypophyseal-adrenocortical system, he postulated that, in the presence of an adrenal cortex under ACTH stimulation, growth hormone acted either by "conditioning" the adrenal cortex to selectively secrete mineralocorticoids, or by "sensitizing" the tissues of the body to the effects of the corticoids produced by the adrenal cortex, or else that the corticoids "sensitized" the tissues of the body to the growth hormone itself. Hence, it seems reasonable to postulate that the secretion of growth hormone by the anterior lobe of the hypophysis may also be of importance in explaining the difference in the ability of immature and young mature rats bearing regenerating adrenals to develop hypertension and vascular disease.

SUMMARY

The development of adrenal-regeneration hypertension and cardiovascular-renal lesions has been studied in uninephrectomized immature and young mature Sprague-Dawley rats allowed 1% sodium chloride to drink. By the end of 8 weeks the immature rats had developed marked hypertension and arteriolar lesions of variable severity in the kidney, heart, brain, adrenal capsule, mesentery, pancreas, and submucosa of the gastrointestinal tract. The arteriolar lesions closely resemble the fibrinoid arteriolar necrosis of malignant hypertension in the human being. Arterial changes characteristic of periarteritis nodosa in this species were also present in the mesentery and pancreas of some of the rats. In the same length of time the young mature rats, on the other hand, showed only a slight rise in blood pressure and fibrinoid vascular necrosis was entirely absent.

Several possible explanations for this effect of age on the development of adrenal-regeneration hypertension and vascular disease have been discussed. Although no significant morphologic difference between the appearance of the regenerated adrenals in immature and young mature rats was observed, these experimental results along with the observations of others suggest that either a quantitative or qualitative difference in the secretory capacity of the adrenal cortex may exist in rats of different ages. The possibility also exists that the failure of young mature rats to develop significant hypertension and vascular changes was a reflection of their lower consumption of saline drinking solution. Since adrenal-regeneration hypertension and concomitant vascular disease develop in actively growing rats but not in older animals, it is suggested that growth hormone may play a significant role in the genesis of this form of experimental hypertension and vascular disease in the rat.

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EFFECT OF THYROXINE ON THE LATENT APYRASE ACTIVITY OF CANARY LIVER MITOCHONDRIA

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RECENT biochemical studies on the action of thyroid hormones have indicated that such hormones as thyroxine and triiodothyronine uncouple the oxidative phosphorylation in mammalian liver mitochondria *in vitro* and *in vivo* (1, 2, 3, 4, 5). It is also well known that 2,4-dinitrophenol (DNP) is the strong uncoupling agent and it activates strikingly the latent apyrase[2] activity of liver mitochondria (6, 7). Therefore it may be expected that thyroxine activates the enzyme action. In this respect, Lardy and Maley (5) have already observed an acceleration of ATP breakdown in aged rat liver mitochondria by a number of thyroid hormones *in vitro*.

This present investigation was undertaken to observe the effect of thyroxine on the latent apyrase activity of fresh liver mitochondria. We used mouse serum in this experiment, because Klemperer (2) succeeded in demonstrating the *in vitro* uncoupling effect of thyroxine in normal rat liver mitochondria by introducing sheep serum into the reaction mixture. This effect of thyroxine had not been observed in normal rat liver mitochondria in other laboratories which did not use the serum.

EXPERIMENTAL

Mitochondria were prepared from the liver of fed canary, which is a different animal from that used in other laboratories, by the method of Schneider and Hogeboom (8). The initial residue of nuclei and cellular debris were not re-extracted. Usually the mitochondria from 15 mg. wet weight of liver, which contained about 0.12 mg. nitrogen, were used in a single enzyme test.

The reaction mixture consisted of 0.5 ml. of 0.1 *M* tris(hydroxymethyl)-aminomethane buffer, 0.2 ml. of 1.25 *M* sucrose, 0.2 ml. of 0.02 *M* potassium ATP (Schwartz Laboratories) and 0.2 ml. of thyroxine or control solution. The total volume was made up to 1.5 ml. with distilled water. In the case of direct incubation, the enzyme reaction was started by the addition of 0.3 ml. of mitochondrial suspension in 0.25 *M* sucrose. In the case of pre-incubation, ATP was added in order to start the enzyme action, after the mixture without ATP being kept for 30 minutes at 0° C at pH 7.4. After ten minutes' incubation of the reaction mixture at 30° C at pH 7.0 with occasional shaking, 0.5 ml. of 34% trichloroacetic acid was added to stop the enzyme action. 1.0 ml. aliquot of the supernatant was assayed for inorganic orthophosphate (P) by the isobutanol-extracting method, using ascorbic acid as the reducing agent, as described by Takahashi (9). Controls without substrate or enzyme were always run in parallel.

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[2] Here the term "apyrase" refers to the common name of enzymes which catalyze the hydrolytic splitting of adenosinetriphosphate (ATP).

DL-Thyroxine (The British Drug Houses Ltd.) was dissolved in 0.5 ml. of 0.02N KOH and to this 1.25 ml. of water, 0.25 ml. of mouse serum and then 0.5 ml. of 0.02N HCl were added. The final concentration of thyroxine was $1.5\times10^{-3}M$. In a few runs, distilled water was added instead of serum. Control solutions lacked thyroxine in both cases with and without serum. To dilute the thyroxine solution, the control solutions were employed. Preliminary tests have revealed that 0.2 ml. of the control solution containing mouse serum showed no apyrase activity.

RESULTS

Effect of thyroxine on the apyrase activity in the absence of mouse serum. As shown in Table 1, thyroxine inhibited considerably the apyrase activity of canary liver mitochondria, when directly incubated. This inhibition, however, decreased when the mitochondria were pre-incubated for 30

TABLE 1. THE APYRASE ACTIVITY OF CANARY LIVER MITOCHONDRIA DIRECTLY INCUBATED OR PREINCUBATED WITH THYROXINE AND 2,4-DINITROPHENOL

Additions	Micrograms P liberated	
	Direct incubation	Preincubation
Control	5.0	5.5
$7\times10^{-5}M$ thyroxine	2.8	4.2
$7\times10^{-6}M$ thyroxine	4.0	5.8
$1\times10^{-4}M$ dinitrophenol	31.8	24.8
$''+7\times10^{-5}$ thyroxine	25.6	22.1

For explanation see text.

minutes at 0° C at pH 7.4 without ATP. In this case, thyroxine in low concentrations such as $5\text{–}7\times10^{-6}M$ enhanced the apyrase activity consistently, although the rise was only 10% or a little more (Table 2). However even when preincubated, thyroxine in concentrations higher than about $2\times10^{-5}M$ depressed the activity by more than 20%. Under all conditions described above, $1\times10^{-4}M$ DNP activated the apyrase activity 4 to 6 times the control.

Effect of thyroxine on the apyrase activity in the presence of mouse serum. Thyroxine in concentrations up to 2×10^{-4}M caused an appreciable increase in the enzymatic hydrolysis of ATP, the maximal activation being 40%. A typical result is shown in Table 3. The addition of $6\times10^{-3}M$ $MgCl_2$ abolished this activation by thyroxine. The antagonistic action of Mg ions on the thyroxine effect has been observed by Bain (10) and also by

TABLE 2. THE APYRASE ACTIVITY OF CANARY LIVER MITOCHONDERIA PREINCUBATED WITH THYROXINE OR 2,4-DINITROPHENOL

Additions	None	Thyroxine					DNP
Concentration ($\times10^6$)		2	7	12	17	34	100
Apyrase activity*	5.8	5.8	6.6	5.6	4.1	3.5	23.3

* Given in micrograms P liberated.
For explanation see text.

Tapley, Cooper and Lehninger (11). It is to be noted that thyroxine did inhibit the apyrase activity in the presence of serum, when directly incubated, as well as in the absence of serum. In the presence of serum, DNP activated the apyrase activity 7 to 10 times the control one (Table 3).

TABLE 3. EFFECT OF THYROXINE AND 2,4-DINITROPHENOL ON THE APYRASE ACTIVITY OF CANARY LIVER MITOCHONDRIA PRETREATED WITH MOUSE SERUM

Concentration of thyroxine added	Micrograms P liberated	
	Without Mg	With Mg*
$\times 10^{-5} M$		
0	3.4	11.7
1	3.7	12.0
2	4.1	11.9
5	4.8	11.0
10	5.1	11.6
20	5.2	11.4
$1 \times 10^{-4} M$ DNP	47.5	—

* $6.6 \times 10^{-3} M$ $MgCl_2$.
For explanation see text.

Sodium azide in $1 \times 10^{2} M$ lowered the thyroxine-activated apyrase activity to the control level. Azide alone in $1 \times 10^{-3} M$ enhanced twice the intact apyrase action, while depressing completely the DNP activation. Those observations confirm the findings of Robertson and Boyer (12) on the actions of DNP and azide in rat liver mitochondria.

DISCUSSION

According to the current biochemical concept (cf. Lardy and Maley, 5), one of the modes of action of thyroid hormone is to regulate the efficiency of oxidative phosphorylation in mitochondria, the most potent energy-supplying system in living matter. Excess doses of thyroid hormones really uncouple the phosphorylation from the oxidative process (3).

In the present investigation, it has been found that thyroxine caused a moderate acceleration of the enzymatic breakdown of ATP by canary liver mitochondria under rather specific conditions, i.e., after pre-incubated both with and without mouse serum. This effect was more remarkable in the former. However, the extent of this elevation by thyroxine was much less than that caused by DNP. Serum components also augmented the activating effect of DNP upon the apyrase activity. For example, the relative activity was 45.6 and 38.0, when incubated with and without serum respectively.

Since the mitochondria swell after treatment with thyroxine (11, 13) the activation of the latent apyrase of mitochondria by thyroxine may be due to a change in the permeability of the mitochondrial membrane, thus making much of the added ATP accessible to the intra-mitochondrial enzyme. This is also the opinion of the Wisconsin group (H. A. Lardy, personal com-

munication). They showed that a number of thyroid hormones exerts a profound effect on the apyrase activity, using aged rat liver mitochondria (5).

Although in the present study it has also been found that mouse serum components accentuate the ability of thyroxine and DNP to elicit responses in rate of ATP hydrolysis by fresh mitochondria obtained from canary liver, the mechanism of the action of serum components remained obscure. But such an effect may be interesting in considering the action of thyroxine *in vivo*.

SUMMARY

DL-Thyroxine enhanced moderately the latent apyrase activity of canary liver mitochondria *in vitro*, when pre-incubated for 30 minutes at 0° C at pH 7.4 in the presence of mouse serum. The activation by $1–2\times10^{-4}M$ thyroxine was 30–40%, which was less than by $1\times10^{-4}M$ 2,4-dinitrophenol.

In the absence of serum, some slight activation was observed in 5–7 $\times10^{-6}M$. However, regardless of the presence or absence of serum, thyroxine inhibited the apyrase activity considerably when directly incubated.

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INTERRELATIONS BETWEEN THYROXIN AND ADRENALIN IN THE REGULATION OF OXYGEN CONSUMPTION IN THE ALBINO RAT[1]

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THYROXIN and adrenalin are both known to have a calorigenic effect, manifested by an increase in oxygen consumption (1, 2). Ring (3) showed that the response to adrenalin was potentiated by thyroxin, and that the logarithm of the response was proportional to the basal metabolism. That this was a direct relationship between the two hormones was demonstrated by the inability of non-thyroid metabolic stimulants and depressants, such as dinitrophenol and fasting, to affect the response to adrenalin. Schaeffer and Thibault (4, 5) also found that thyroxin greatly increased the response to adrenalin. The potentiation could be observed even before and after the calorigenic effect of the thyroxin itself was evident. In both investigations single large doses of thyroxin were used.

Ring proposed that either a reversible chemical reaction between thyroxin and adrenalin or adsorption may be involved. Recent data on the action of hormones at the enzyme level suggest that adrenalin and thyroxin may influence consecutive rate-limiting reactions, adrenalin by increasing the amount of substrate, i.e., glucose (6, 7), and thyroxin by accelerating the utilization of glucose (8, 9, 10, 11). There is also the possibility that thyroxin prolongs the action of adrenalin by retarding its oxidation by amine oxidase, as thyroxin has been shown to decrease the level of this enzyme (12, 13, 14).

A more intensive study of the interaction of these two hormones at physiological levels of thyroxin, might clarify some of these theoretical considerations, and be useful in defining the relative roles of the thyroid gland and the adrenal medulla in cold adaptation. The present experiment was therefore designed to calibrate the increase in oxygen consumption following a standard adrenalin dose administered to thyroidectomized rats receiving various amounts of thyroxin.

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METHODS

Forty-eight male Sprague-Dawley rats weighing approximately 100 gm were thyroidectomized, and twelve additional rats were kept intact. Two weeks after thyroidectomy, the animals were divided into four groups receiving daily subcutaneous injections of 0, 3, 12, and 48 μg of thyroxin per animal.[2,3] All animals not receiving thyroxin were injected with saline solution. Fifteen days after the start of the injections, the dose of 48 μg was reduced to 24 μg, because the larger dose appeared to be toxic. One set of oxygen consumption measurements was taken at the 48 μg.-level, and five at 24 μg. These will be reported separately.

All animals were kept at 26° C. ± 2° and fed the iodine and thyroxin-deficient diet described by Eartly and Leblond (15). All thyroidectomized animals were given 1% calcium lactate as drinking fluid for 2 weeks postoperatively.

Oxygen consumption was measured 15–60 days after thyroidectomy in an apparatus similar to that described by Grad (16). Animals were conditioned to the apparatus before use, and were allowed to rest in the glass jars in which their oxygen consumption was taken for 1 hour prior to measurements. The water bath was kept at 28° C. ± 0.5°, and the temperature inside the bottles was 30° C. ± 1°.

In the first experiment, several doses of adrenalin[4] were tested on intact and thyroidectomized animals in order to determine the relative potency, and to choose a suitable test dose. Oxygen consumption was measured before administration of adrenalin and then 15 minutes, 1 hour, 2 hours, 3 hours and 4 hours after a single intra-muscular injection of 40, 80, or 160 μg. of adrenalin/100 gm. body weight.[5] Several days were allowed to elapse between the testing of the various doses.

Since 40 μg. of adrenalin gave an adequate response, which was maximal after approximately 1 hour, the following procedure was routinely used to measure the oxygen consumption response to adrenalin of rats maintained at various levels of thyroxin: (1) The oxygen consumption without adrenalin was measured in each group. (2) After suitable time intervals, the animals in each group received 40 μg. of adrenalin intramuscularly. (3) One hour after injection of adrenalin, the oxygen consumption was again measured.

The same procedure was repeated with 100 μg. of adrenalin, but this dose proved fatal to animals receiving more than 3 μg. of thyroxin. The experiment was repeated with substantially similar results, which will be reported in a subsequent publication, in conjunction with observations made at different temperatures.

RESULTS

Oxygen consumption after adrenalin. All three doses of adrenalin (40, 80, and 160 μg. caused a marked increase in oxygen consumption by intact rats (Fig. 1). The maximum response was observed after 1 hour, and the effect gradually diminished, consumption returning to normal by 4 hours. The magnitude, but not the duration, of the response varied directly with the dose of adrenalin. On the other hand, the oxygen consumption of thyroidectomized rats was not influenced by adrenalin.

Oxygen consumption by thyroidectomized rats after thyroxin. Two weeks

[2] All doses of thyroxin will subsequently be expressed in μg./animal daily.

[3] Squibb—90% l-thyroxin, in slightly alkaline saline solution.

[4] Parke-Davis, 1 cc. ampoules of adrenalin-in-oil 1:500. Adrenalin-in-oil was found preferable to adrenalin chloride because its slower absorption prevented local necrosis.

[5] All doses of adrenalin will subsequently be expressed as μg./100 gm. body weight.

after operation, the oxygen consumption by all thyroidectomized rats had dropped to 0.81 of that by the intact controls. Daily injections of thyroxin were now begun; the oxygen consumption rose in proportion to the daily dose of thyroxin and reached a plateau in 5 days. The oxygen consumption varied linearly as the logarithm of the thyroxin dose (Fig. 2). This relationship remained fairly constant during the 24-day experimental period.

Average results and standard error of six measurements taken on 5 rats

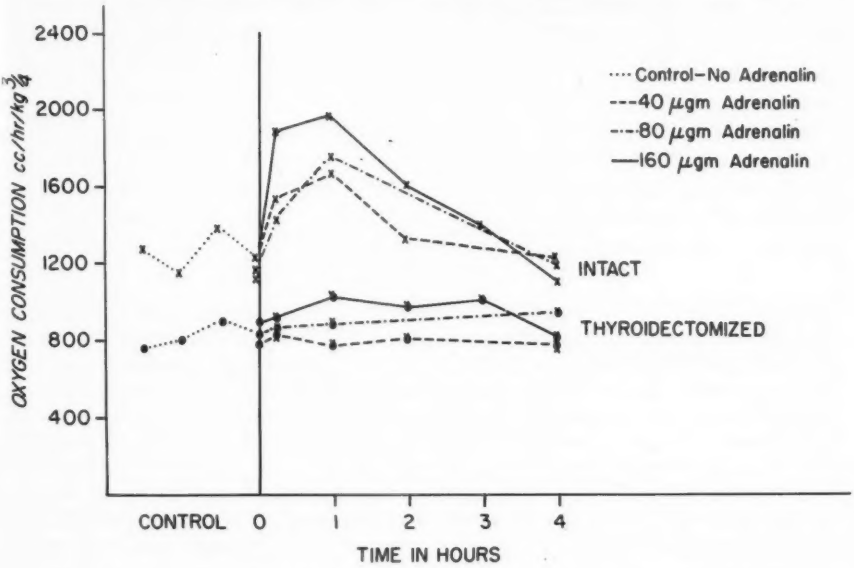

FIG. 1. Time response of intact and thyroidectomized rats to single injections of adrenalin (40, 80, or 160 μg./100 gm.). Thyroidectomized rats showed no significant response to adrenalin over a 4-hour period. Intact rats immediately responded to various doses of adrenalin by proportional increases in oxygen consumption, maximal at 1 hour and no longer observable at 4 hours.

each are shown in Table 1. Differences between all groups were significant by analysis of variance and the "t" test. However, since only one measurement of 5 animals was taken at 48 μg., the results were not significantly different from those obtained with 24 μg. Three, twelve, twenty-four and forty-eight μg. of thyroxin elevated the metabolism above the level of the thyroidectomized 1.31, 1.55, 1.67 and 1.75 times respectively. The oxygen consumption of the intact controls was 1.22 times that of the thyroidectomized.

Oxygen consumption by thyroidectomized rats after thyroxin and adrenalin. Intact controls and all thyroxin-treated animals responded to injection of 40 μg. of adrenalin with a marked rise in oxygen consumption (Table 1, Fig. 2). The increment of increase was proportional to the original level of

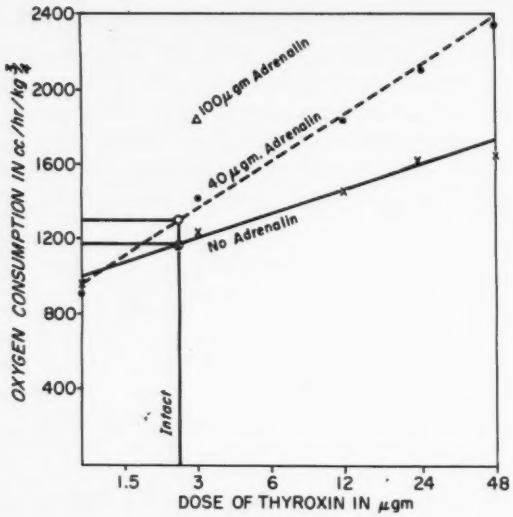

FIG. 2. Regression of oxygen consumption of thyroidectomized rats treated with various doses of thyroxin plotted against the dose of thyroxin on a logarithmic scale. For the sake of completeness, the thyroidectomized animals were considered to secrete 1 μg. of thyroxin. This is justified by the observation of Leblond and Eartly (17) who postulated a minimal amount of circulating thyroxin even in completely thyroidectomized rats on iodine-deficient diet. I. Without adrenalin the slope was 0.31. II. One hour after 40 μg. of adrenalin per 100 gm. body weight, the slope was 0.62. III. No curve could be obtained with 100 μg. of adrenalin, because this dose was fatal to all animals receiving more than 3 μg. of thyroxin. ⊗ and ○ represent the oxygen consumption of intact controls without and with adrenalin, respectively.

TABLE 1. OXYGEN CONSUMPTION OF INTACT AND THYROIDECTOMIZED RATS, RECEIVING THYROXIN AND ADRENALIN

Group	Daily dose thyroxin	Oxygen consumption (cc./hr./kg.$^{3/4}$)			Proportional increase in oxygen consumption				
					After adrenalin		After thyroxin		
		Dose adrenalin			(μg.)		μg. adrenalin		
		0 μg.	40 μg.	100 μg.	40	100	0	40	100
Intact	0	1161 ± 22* 956 ± 25	1304 ± 27	1244 ± 89	1.12	1.30	1.22	1.46	1.58
Operated	0	946 ± 15 562 ± 113	891 ± 20	468 ± 94	0.94	0.83			
Operated	3	1246 ± 21 1284 ± 22	1437 ± 31	1836 ± 42	1.15	1.42	1.31	1.61	2.34
Operated	12	1463 ± 27	1868 ± 41		1.27		1.55	2.01	
Operated	24	1589 ± 28	2108 ± 65		1.32		1.68	2.36	
Operated	48	1651 ± 53	2368 ± 58		1.43		1.75	2.65	

± Standard error.
* All measurements taken 15–60 days after beginning of thyroxin injections. Measurements appearing on the same line were taken the same day.
Statistical Significance:
Differences elicited by thyroxin significant between all doses, except 24 and 48 μg.
Differences elicited by adrenalin significant at all thyroxin doses.
Since the variance between animals and between measurements was not significant, "n" was taken to represent ndividual measurements.

metabolism, and was highly significant in all cases. Adrenalin increased the oxygen consumption of intact controls 1.12 times, 1.46 times the thyroidectomized value. It increased that of animals receiving 3, 12, 24 and 48 μg. of thyroxin 1.15, 1.27, 1.32 and 1.43 times, respectively, i.e., 1.61, 2.01, 2.36 and 2.65 times the thyroidectomized value. On the other hand, the oxygen consumption of thyroidectomized rats was significantly decreased, 0.94 times. The oxygen consumption in the presence of 40 μg. of adrenalin also showed a linear relation to the logarithm of the thyroxin dose (Fig. 2). The intercept is similar to that of the curve without adrenalin, but the slope is doubled.

Later a single dose of 100 μg. of adrenalin was injected into these rats. This caused the oxygen consumption of intact controls and of animals receiving 3 μg. of thyroxin to rise 1.30 and 1.43 times, respectively, or to 1.58 and 2.34 times the thyroidectomized value. Animals on higher levels of thyroxin did not survive this dose of adrenalin. In contrast, the oxygen consumption of thyroidectomized rats was against decreased, this time 0.83 times.

All animals, except the thyroidectomized, responded to adrenalin injection by copious urination and defecation, rapid respiration, and restlessness followed by exhaustion. The symptoms became more severe if the dose of either thyroxin or adrenalin was increased and most severe when the animal was receiving large amounts of both hormones.

DISCUSSION

Oxygen consumption varied linearly with the log dose thyroxin and approached maximum value at 48 μg. of thyroxin daily, at which dose the animals showed signs of thyrotoxicosis. The results compared favorably with those previously obtained with comparable thyroxin doses (15) and with those of other investigators using larger doses for a shorter time (3, 1). Although the oxygen consumption of animals receiving 48 μg. of thyroxin may be close to the physiological limit for a sustained metabolic rate, it could be significantly increased for a short time by adrenalin.

An unexpected observation was that in the absence of endogenous or exogenous thyroxin, adrenalin could not exert its supposed calorigenic effect. In fact, adrenalin produced a slight but significant lowering of oxygen consumption. This is contrary to the results of Ring (3) and of Schaeffer and Thibault (4, 5) who obtained increases of 43.4% and 8.0%, respectively, in thyroidectomized rats. Since dietary iodine and thyroxin were not strictly controlled by these investigators, their thyroidectomized rats may have been eating quantities of thyroxin large enough to mediate adrenalin's effect on oxygen consumption (17).

Intact rats responded to adrenalin by an increase in oxygen consumption proportional to the dose of adrenalin; this finding agrees with Morin's (18) observations on the dog.

The oxygen consumption after a standard dose of adrenalin varied linearly with the log dose thyroxin, the oxygen consumption/thyroxin ratio depending on the adrenalin dose. Thyroxin doubled the effectiveness of 40 μg. of adrenalin, as well as causing 100 μg., to be a fatal dose to animals receiving more than 3 μg. of thyroxin. The increased toxicity confirms Peltola's (19) observation that the administration of thyroid powder to white mice decreased the lethal dose of adrenalin to 1/7. The action of thyroxin in its potentiation of adrenalin is not an all-or-none or threshold effect, as it is with growth hormone (15), but depends on the amount of thyroxin. The results with small daily doses of thyroxin agree with those obtained by Ring (3) and Schaeffer and Thibault (4, 5) with large single doses.

The interpolation of the oxygen consumption value on a thyroxin dose-response curve has been used to estimate the thyroxin requirements of intact rats under various conditions (20). The greater response obtained here with adrenalin suggests its use as a confirmatory measure of thyroid activity. The estimate of daily requirements of intact rats under conditions of this experiment (26° C.±2°) agreed closely whether the oxygen consumption was measured without or with adrenalin. The estimated requirement was 2.5 μg. of l-thyroxin (Fig. 2). This compares favorably with previous findings of 6 μg. of d,l-thyroxin (15) or 5.2 μg. of l-thyroxin (21), and of 3.5–5.0 μg. of l-thyroxin (20).

Although the endogenous secretion of adrenalin could not be controlled, preconditioning the animals to the apparatus and keeping them in a warm room minimized secretion above the resting level. Oxygen consumption measurements were taken at thermal neutrality (22). Endogenous secretion could not have been significant in comparison with the injected dose, since the responses to 40 μg. and to 100 μg. of adrenalin clearly differed. The possibility of endogenous adrenalin secretion must be kept in mind if experimental conditions, such as temperature, are changed.

There is good evidence that daily injections of thyroxin will maintain the level of circulating hormone constant. In practice, it makes no significant difference at what time of day oxygen consumption is measured in relation to the time of thyroxin injection. Gross and Leblond (23) showed that the half-life of a tracer dose of thyroxin is 18 to 24 hours, and that large doses are excreted relatively faster, while Johnson and Albert (24) reported that most of a tracer dose of thyroxin is excreted by 48 hours. Since the metabolic effect of a single large dose of thyroxin does not become evident until 12 hours, and is maximal after several days (25), it seems likely that daily injections of thyroxin will summate to give a smooth level of hormone. Bargeton and Krumm-Heller (26) suggest that the quantity of a drug retained in an organism is proportional to the log of the injected dose. Therefore, if the response to the injected dose is logarithmic, the response to the circulating level is linear. We may therefore assume that the circulating

thyroxin levels remained reasonably constant, even though we do not know their absolute value.

The most attractive theory of the synergistic relationship between thyroxin and adrenalin is that they influence consecutive rate-limiting reactions in the metabolic cycle, thyroxin acting at a later stage than adrenalin. There is a great deal of recent evidence from enzymatic studies that under normal or hypothyroid conditions, the rate of oxidation is partly or completely geared to the demand for high-energy phosphates, and can therefore be increased *in vitro* by the addition of a phosphate acceptor system, e.g., glucose-hexokinase. In thyrotoxic animals the rate of oxidation is very much increased and is no longer influenced by the addition of phosphate acceptors (9, 11). At the same time, the P/O ratio is decreased (although the absolute oxygen and phosphorus uptake is increased) (27, 9, 8). This suggests that thyroxin increases metabolism by partially uncoupling oxidation and phosphorylation, resulting in the inefficient release of heat. It is conceivable that this reaction is rate-limited by the availability of substrate, and that by its glycogenolytic action, adrenalin increases the rate at which substrate is produced. Adrenalin and thyroxin may then have a synergistic action on metabolism in that adrenalin increases the rate of production of substrate and thyroxin increases its utilization. In the absence of thyroxin, the rate is limited by the utilization of high-energy phosphate, and cannot be influenced by an increase in substrate. Adrenalin has therefore no calorigenic effect in thyroidectomized rats.

In addition to this mechanism, thyroxin may also retard the breakdown of adrenalin, so that it has more time to exert its effects, resulting in summation. Leduc *et al.* (14) showed that thyrotoxic animals retained more exogenous adrenalin in the tissues. This would explain why thyroxin also facilitates many non-calorigenic effects of adrenalin, e.g., hypertension (28) and hyperglycemia (13) *in vivo* and inhibition of intestinal tone, contration of spleen and uterus (29), increased rate and metabolism of heart (30), and vasoconstriction of arteries (31) at the perfused organ level. According to Lund (32), there are three pathways for the elimination of adrenalin: (1) Complete destruction by oxidative deamination in the liver (most important), (2) slower oxidation to adrenochrome by cytochrome oxidase in muscles, and (3) excretion, as adrenalin sulphuric acid ester, through the kidneys. Thyroxin may retard adrenalin catabolism by decreasing the level of amine oxidase in the liver (12). Several effects of adrenalin which are stimulated by thyroxin, e.g., arterial vasoconstriction (31) and hyperglycemia (13), are also stimulated by ephedrine, a known amine oxidase inhibitor.

On the other hand, Bacq (33) believes that thyroxin, like other phenols which sensitize tissues to adrenalin (catechols, hydroquinone, pyrogallol), acts as an anti-oxidant in slowing the oxidation to adrenochrome, and Thi-

bault (29) demonstrated this effect *in vitro*. Through whichever mechanism thyroxin retards the elimination of adrenalin, accelerated destruction alone cannot account for the complete inactivation of adrenalin in thyroidectomized animals. Synergism at the metabolic level and prolongation of adrenalin action play a part in calorigenic potentiation of thyroxin.

SUMMARY

Thyroidectomy inhibited and thyroxin potentiated the calorigenic effect of adrenalin. The oxygen consumption of thyroidectomized rats receiving daily doses of 3, 12, 24 and 48 μg. of thyroxin per animal varied linearly as the log dose thyroxin. One hour after intra-muscular injection of 40 μg./100 gm. body weight of adrenalin, the oxygen consumption was increased by increments proportional to the log dose thyroxin. The oxygen consumption after adrenalin still varied linearly as the log dose thyroxin, but the slope was approximately doubled. The sensitivity to adrenalin was so increased by thyroxin, that a dose of adrenalin (100 μg./100 gm. body weight) tolerable to intact or thyroidectomized animals was fatal to those animals receiving more than 3 μg. of thyroxin. Intact rats responded to adrenalin injection by an increased oxygen consumption proportional to the dose of adrenalin. The effect was evident at 15 minutes, maximal at 1 hour, and no longer observable at 4 hours. The response of intact rats to adrenalin indicated a level of thyroxin consistent with that indicated by the oxygen consumption without adrenalin.

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LIFE MAINTENANCE AND MINERALOCORTICOID ACTIVITY OF SOME NEW DERIVATIVES OF CORTISONE, HYDROCORTISONE AND DOC[1]

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NEW compounds representing interesting modifications of the structure of cortisone, hydrocortisone and desoyxcorticosterone have appeared within recent years. Some of these compounds exhibit physiological properties and potencies which are lacking in the parent steroids. Thus halogenation at the 9 alpha position of hydrocortisone markedly enhances both gluco- and mineralocorticoid activity of this steroid (1). Dehydrogenation of hydrocortisone at positions C-1 and C-2 definitely exaggerates the glucocorticoid function, but the sodium retaining activity, as shown by tests on rats (2, 3), and clinical studies on patients (4, 5, 6, 7), appears to be considerably diminished. When, however, chemical modifications such as halogenation at the 9 alpha position and dehydrogenation at C-1 and C-2 are both present in the same molecule, the biological activity of the cortisones is greatly altered with respect to both gluco- and mineralocorticoid activity (8, 9). The present investigation is concerned with the effect of gradual reduction of dosage of several of these modified corticoids upon life maintenance, blood pressure and serum electrolytes of adrenalectomized dogs.

METHODS

The compounds tested were 1-dehydro derivatives of cortisone, hydrocortisone and desoxycorticosterone which were prepared in the laboratories of the Schering Corporation, Bloomfield, New Jersey, and 1-dehydro-9α-fluorohydrocortisone acetate which was obtained from Dr. Joseph Fried of the Squibb Institute for Medical Research, New Brunswick, New Jersey.

The test animals were adrenalectomized dogs which had been without adrenals for 2–5 years and had served repeatedly for study of various corticoids. They had been subjected to numerous bouts of adrenal insufficiency and recovery and during the intervals between tests were adequately maintained on daily doses of 0.5 mg./dog/day of DCA plus addition of salt supplements.

Three days before beginning of an experiment the salt was withdrawn and a diet containing a total of 1.47 gm. of Na and 0.94 gm. of K was substituted. The diet and other procedures used for assay studies on adrenalectomized dogs have been reported

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elsewhere (9). Each dose level was maintained for 10 days. Arterial pressure, blood samples, etc., were then taken and the daily dose of steroid reduced by 50% until either frank symptoms of adrenal insufficiency appeared or changes occurred in the blood constituents indicative of impending adrenal failure. Since none of the steroids employed were water soluble in significant amounts, the crystalline materials were dissolved in warm 95% ethanol and the daily dose withdrawn and diluted dropwise with warm water to a 15% solution for injection. The steroids were administered subcutaneously in divided doses, A.M. and P.M. Adequate references to methods used for determining arterial pressure, serum electrolytes and other blood constituents have been reported (10).

RESULTS

1-dehydrohydrocortisone. The data in Table 1 reveal that adrenalectomized dogs cannot be maintained with normal serum electrolytes while receiving daily doses of 10 mg./dog/day. Doses of 1–5 mg./day were tested but found to be grossly inadequate, and severe serum electrolyte changes occurred during the first 10 days of the experiment. Even when the dose was raised to 10 mg./day the blood urea nitrogen increased by 15–20 mg. and there was a pronounced decline in serum Cl in the two dogs studied. One animal developed a severe diabetes-insipidus-like syndrome, which will be referred to hereafter as DIS (Fig. 1). The other animal at no time ingested as much as 500 cc. H_2O. When the dose was increased to 15 mg./day the single test dog showed but a few m.eq/l reduction in serum

TABLE 1. MAINTENANCE REQUIREMENTS OF THE ADRENALECTOMIZED DOG FOR 1-DEHYDROCORTISONE AND 1-DEHYDROHYDROCORTISONE

Dose/ dog/day	Exper. Period	Wt.	B.P.	Blood Urea N.	Hb.	Hmct.	R.B.C.	Blood Sugar	Serum Na	Serum Cl	Serum K
milli-grams	days	Kg.	mm.Hg.	mg.%	gm.%	%	10^6/ cu. mm.	mg.%	m.eq./L.	m.eq./L.	m.eq./L.
1-dehydrocortisone											
	Initial	10.88	116	18.3	11.82	34.3	6.50	81.5	138.5	110.0	4.72
10.0	10	9.98	101	33.0	12.40	36.6	6.80	86.0	127.0	92.3	5.60
	Initial	18.86	105	19.7	13.89	36.2	6.30	72.5	140.5	115.5	4.22
15.0	10	17.80	112	31.5	12.48	38.5	5.97	97.5	137.0	105.6	5.10
7.5	10	13.35	91	38.5	15.2	34.0	5.79	107.5	127.0	90.9	5.85
1-dehydrohydrocortisone											
	Initial	11.10	115	20.9	12.05	34.2	6.56	75.0	140.0	116.9	4.06
10.0	10	10.43	125	35.5	12.28	39.2	6.45	105.0	137.5	102.5	5.14
5.0	10	10.21	105	31.2	13.78	34.7	6.50	99.5	138.0	109.4	4.93
2.5	10	10.43	130	44.0	13.33	30.2	5.80	118.5	131.0	93.6	5.85
	Initial	12.93	115	17.7	13.33	33.2	4.77	90.0	145.0	114.1	5.20
10.0	10	12.36	120	37.7	13.61	33.1	4.78	108.0	140.0	101.0	5.95
5.0	10	12.48	109	64.6	14.38	32.3	4.66	110.0	120.0	87.0	7.96
	Initial	14.40	98	24.5	12.17	31.0	5.35	82.5	140.5	116.5	4.26
15.0	10	13.72	112	20.5	12.28	30.7	5.40	96.0	137.5	100.0	4.28
7.5	10	13.15	113	27.5	13.05	31.7	5.77	115.0	124.5	90.5	4.71
	Initial	14.85	100	20.0	12.27	29.3	5.10	82.5	146.5	116.2	4.64
20.0	10	13.72	117	19.5	13.47	32.7	5.85	99.5	142.0	104.4	4.77
10.0	10	13.61	117	18.0	12.05	31.2	5.44	101.0	129.5	93.6	4.46
5.0	8	13.84	119	26.0	14.03	31.1	6.02	106.0	122.0	82.0	5.44
	Initial	17.58	108	19.0	12.91	30.6	4.80	84.0	145.5	115.0	4.48
20.0	10	15.99	131	23.5	14.03	32.2	5.42	90.0	141.0	97.4	5.35
10.0	10	15.31	129	41.3	14.03	32.5	5.33	106.0	135.0	93.3	5.48
5.0	8	14.85	118	64.0	16.46	37.0	6.32	100.0	133.0	94.0	7.37

Doses italicized are minimum maintenance levels.

Na but a striking fall in serum Cl. Lowering the dose to 7.5 mg./day induced a drastic change in both Na and Cl. Throughout the test the water intake slowly increased from the normal level of 350 cc. to 2500 cc./day.

Two dogs were given an initial dose of 20 mg./dog/day of the steroid, but even this large amount proved to be inadequate for maintaining a normal serum Cl. Reduction of the dose to 10 mg./day led to loss of both Na and Cl. One animal did not increase his water intake even when given a dose of 10 mg./day for 10 days followed by 10 mg./day—for an additional 7 days (Fig. 1). It is of interest that the mean arterial pressure of all of the dogs receiving this compound increased moderately during the interval the animals were injected with the higher dosage. Furthermore, it will be observed that even when the serum Na and Cl were at low levels the blood pressure remained within the normal range. None of the dogs exhibited symptoms, remaining active and vigorous in spite of the lowered serum Na and Cl. The serum K became elevated only when the dose level was sharply reduced and the serum Na had fallen.

1-dehydrocortisone. Four dogs were tested on this compound at initial doses of 1, 5, 10 and 15 mg./day, but even the animal receiving 15 mg./day was unable to maintain a normal serum electrolyte pattern and blood urea nitrogen for more than a few days. Therefore, the maintenance dose of the compound was considered to be approximately 20 mg./day, since changes occurred in the blood constituents at the 15 mg. dose level which were indicative of inadequate dosage (Table 1). Representative data from 2 of the animals are given in Table 1.

1-dehydrodesoxycorticosterone. DOC that had been subjected to dehydrogenation at C-1 and C-2 displayed little, if any, alteration in physiological activity from the parent steroid. Four dogs receiving the material were studied, and since there are no reports in the literature pertaining to use of this steroid, the initial dose was arbitrarily set at 0.5 mg./day. The data obtained from tests on 2 of the 4 animals are recorded in Table 2, where it will be noted that the minimum maintenance dose was approximately 250 μg./day. The arterial pressure slowly declined when the dose level fell below 250 μg./day. However, one dog showed some increase in serum K at this dose.

1-dehydro-9α-fluorohydrocortisone. This steroid was assayed for mineralocorticoid activity on 4 dogs. Substitution of a fluorine atom at the 9 alpha position of hydrocortisone transformed a substance of low Na-retaining activity into an extremely potent corticoid which ranks with aldosterone insofar as ability to maintain life and a normal serum electrolyte pattern at low dosage is concerned. The maintenance dose was 6.25 μg./dog/day for 3 animals and 12.5 μg./day for one. The failing dose at which unequivocal alterations in serum K and blood pressure appeared was 3.2 μg./day. It had been demonstrated in previous experiments (11), that the maintenance dose of aldosterone was 6.2–12.5 μg./day. These figures are identical

TABLE 2. MAINTENANCE REQUIREMENTS OF ADRENALECTOMIZED DOGS FOR 1-DEHYDRODESOXYCORTICOSTERONE AND 1-DEHYDRO-9α-FLUOROHYDROCORTISONE

Dose/dog/day	Exper. period	Wt.	B.P.	Blood urea N.	Hb.	Hmet.	R.B.C.	Blood sugar	Serum Na	Serum Cl	Serum K
micrograms	days	Kg.	mm.Hg	mg.%	gm.%	%	10^6/cu. mm.	mg.%	m.eq./L.	m.eq./L.	m.eq./L.
1-dehydrodesoxycorticosterone											
	Initial	10.99	125	23.5	11.48	34.1	6.24	87.5	138.5	110.0	5.11
500	10	11.57	121	17.2	12.17	30.6	5.96	72.5	143.0	110.0	4.72
250	10	11.79	114	23.9	13.05	28.5	6.33	85.0	138.5	107.3	6.11
125	10	11.57	92	35.0	13.19	34.5	6.42	84.5	140.0	107.0	6.43
62.5	1	11.79	83	58.0	13.33	33.4	5.90	86.0	137.0	105.9	6.50
	Initial	10.66	118	22.5	11.00	33.3	5.45	85.0	142.5	115.5	4.22
500	10	10.99	103	18.2	11.48	27.6	5.21	80.5	145.0	112.1	4.12
250	10	10.66	98	25.6	11.82	31.6	5.65	83.0	143.0	111.0	4.87
125	10	11.10	85	30.0	13.47	34.8	6.00	83.5	142.0	110.0	6.01
62.5	5	10.43	65	54.5	13.33	35.3	6.30	81.0	137.5	108.0	6.30
1-dehydro-9α-fluorohydrocortisone											
	Initial	14.06	110	13.4	13.8	33.1	5.5	77.0	143.0	113.5	4.00
50.0	10	13.9	130	18.7	11.8	30.6	4.7	90.5	146.3	114.1	4.12
25.0	10	13.8	112	16.5	12.2	37.6	4.4	84.5	152.5	118.2	4.79
12.50	10	14.06	102	15.8	14.9	41.8	6.2	85.0	146.0	110.0	5.03
6.25	10	14.1	108	17.9	17.3	44.9	7.3	88.0	140.5	108.0	5.85
3.20	10	13.7	100	30.6	17.5	48.9	7.9	81.5	137.0	112.0	7.21
	Initial	16.3	108	17.7	12.6	31.1	5.1	85.0	145.0	114.0	3.90
50.0	10	16.1	120	17.8	12.9	35.5	5.7	82.5	152.5	115.5	4.90
25.00	10	15.9	124	20.4	13.1	37.9	6.5	83.5	154.5	122.3	5.13
12.50	10	16.5	110	19.8	14.3	39.2	5.8	87.0	148.5	113.5	4.95
6.25	10	16.6	109	24.8	18.3	48.1	6.3	82.5	145.5	114.0	6.17
3.20	10	16.4	102	26.0	17.1	49.4	7.1	84.5	142.0	113.8	6.82
	Initial	13.3	100	22.4	11.4	31.8	5.3	76.5	141.1	114.9	4.54
25.00	10	13.1	138	17.7	12.4	32.3	4.9	80.0	145.0	111.0	4.47
12.50	10	12.5	108	18.5	12.7	37.1	5.7	78.5	149.5	114.0	4.96
6.25	10	12.1	102	15.0	14.7	38.0	5.7	79.5	149.5	117.0	5.03
3.20	10	12.8	96	27.0	18.4	37.0	7.0	82.0	145.0	113.5	6.76
	Initial	15.0	102	21.0	12.4	33.6	4.9	90.0	146.0	114.5	4.50
25.00	10	14.8	130	16.5	13.0	32.6	5.2	92.0	149.0	114.8	4.87
12.50	10	14.5	106	17.4	17.0	41.5	6.5	80.0	148.5	116.0	6.18
6.25	10	14.6	94	19.4	15.7	41.0	6.0	85.0	144.0	110.0	6.34
3.20	10	14.6	72	87.0	15.4	42.8	6.1	87.0	141.0	113.0	9.85

Doses italicized are minimum maintenance levels.

with those obtained for 1-dehydro-9α-fluorohydrocortisone in the present experiments.

An interesting feature of the response to daily injections of this halogenated compound was the slow increase in the mean arterial pressure which followed administration of 50–25 μg./day. The initial resting pressure of this series of table-trained dogs varied between 100–110 mm. Hg, but they developed a sustained pressure increase of 16–38 mm. Hg which slowly declined as the dose was reduced.

The serum Na and Cl were maintained at normal levels on a daily dose of 3.2 μg./day, but these electrolytes increased markedly in 3 animals on the higher doses (Table 2). The Na and Cl did not decline below normal even when it was necessary to terminate the experiments owing to retention of serum K. The behavior of this cation contrasted sharply with that of Na, for in all cases studied the K accumulated as the steroid dosage was lowered whereas the Na and Cl remained unchanged. One dog exhibited a value for serum K of 9.85 m.eq/l and died less than 24 hours after institution of vigorous replacement therapy with DCA and cortisone suspensions.

Blood concentration was present in all of the animals as indicated by increases in hemoglobin, hematocrit and R.B.C.

The effect of 1-dehydrohydrocortisone and 1-dehydro-9α-fluorohydrocortisone upon the water balance was studied in 4 dogs, two for each compound. The pertinent data are summarized in Figure 1. An intimate association apparently exists between the greatly elevated water intake and the high serum Na in animals receiving 1-dehydro-9α-fluorohydrocortisone. Severe DIS developed in one animal on 1-dehydrohydrocortisone and the disturbed water balance was accompanied by an actual decrease in the

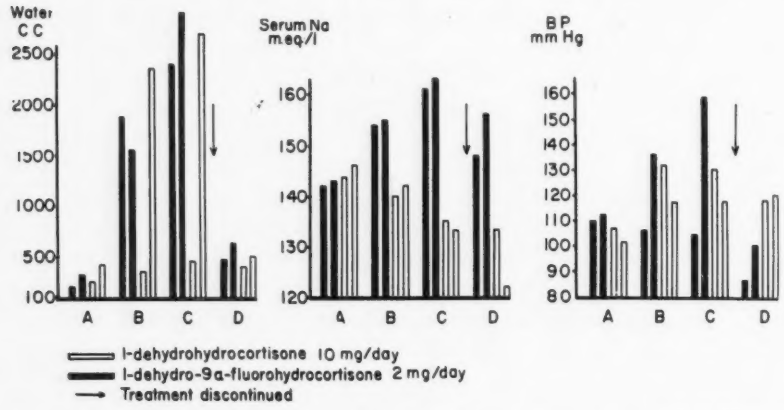

FIG. 1. Water consumption, serum sodium and blood pressure of four adrenalectomized dogs, two of which received 1-dehydrohydrocortisone and two 1-dehydro-9α-fluorohydrocortisone. A: Fore period 6 days. B and C: 17 days steroid treated. D: 6 days after discontinuing treatment.

serum Na. It was also observed that one dog injected with 1-dehydro-9α-fluorohydrocortisone exhibited DIS plus high serum Na but with no accompanying increase in the mean arterial pressure (Fig. 1).

DISCUSSION

The experiments demonstrate that large doses of 1-dehydrocortisone and 1-dehydrohydrocortisone are required for maintenance of a normal serum electrolyte pattern of adrenalectomized dogs. Dehydrogenation at C-1 and C-2 greatly reduces the Na-retaining properties of these steroids thus they must be administered in doses of 20 mg./dog/day. This amount is four times the dose that is necessary for maintenance when the chemically unaltered cortisones are used under the conditions of these experiments. This is a surprising dosage requirement when it is recalled (Table 3), that dogs are well maintained on 5 mg./day of cortisone and hydrocortisone, 250 μg./day of DOC, 6–12.5 μg./day of aldosterone, 6–12.5 μg./day of 1-dehydro-9α-fluorohydrocortisone and 6.8–13.7 μg. of fluoro-corticosterone

acetate (11, 12). Although dehydrogenation of the cortisones at C-1 and C-2 lowers the ability of the steroids to retain Na, they still exhibit strong glucocorticoid activity according to Perlman and Tolksdorf (2), whereas Bunim, *et al.*, (4, 5), clearly demonstrated their value as therapeutically active compounds for treatment of rheumatoid arthritis.

A comparison of the data shown in Table 1 with those in Table 2 reveal interesting biological differences between the dehydrogenated cortisones and similar modification of DOC. The serum Na and Cl show a drastic

TABLE 3. RELATIVE POTENCY OF SOME NATURALLY OCCURRING AND CHEMICALLY MODIFIED ADRENAL STEROIDS

Non-halo compounds	Dogs tested	MM dose/day*	Potency DOC = 1
Aldosterone	4	6.25– 12.5 μg.	20–40.0
Desoxycorticosterone	4	125.00–250.0 μg.	1.0
1-dehydrodesoxycorticosterone	4	250.0 μg.	1.0
Cortisone	4	5.0 mg.	0.05
Hydrocortisone	4	5.0 mg.	0.05
1-dehydrocortisone	4	20.0 mg.	0.0125
1-dehydrohydrocortisone	4	20.0 mg.	0.0125
9α-halo compounds			
Fluoro-hydrocortisone acetate	3	27.5 μg.	9.1
Chloro-hydrocortisone acetate	5	55.0 –110.0 μg.	2.3– 4.5
Fluoro-corticosterone acetate	4	6.8 – 13.7 μg.	18.0–36.0
1-dehydro-9α-fluorohydrocortisone	4	6.2 – 12.5 μg.	20.0–40.0
Fluoro-11β-hydroxyprogesterone	2	150.0 μg.	1.67
Fluoro-11β-17α-dihydroxyprogesterone	2	300.0 μg.	0.83

*MM = minimum maintenance dose.

decline when the dogs are receiving large doses (10–15 mg./day) of the 1-dehydrocortisones whereas these electrolytes were well maintained in the animals receiving 250 μg./day of the DOC derivative. This minimum dose level is identical with that obtained in tests made several years ago using unmodified DOC. A summary of the relative potencies of the various steroids that have been tested in this laboratory for their mineralocorticoid activity in adrenalectomized dogs is given in Table 3. The standard for comparison is DOC, and all compounds were solubilized in 10–15% ethanol and administered in identical fashion.

Aldosterone and 1-dehydro-9α-fluorohydrocortisone proved to be the most potent steroids tested, with fluoro-corticosterone a close second. The least effective compounds for Na-retention were the 1-dehydrocortisones.

SUMMARY

Four corticoids representing chemical modifications of cortisone, hydrocortisone and desoxycorticosterone were studied for their effects on life maintenance and mineralocorticoid activity on adrenalectomized dogs. 1-dehydrocortisone and 1-dehydrohydrocortisone proved to be quite low in the Na retaining activity since the minimum maintenance dose required

to maintain a normal serum electrolyte pattern was 20 mg./dog/day, which is five times the minimum maintenance dose of cortisone or hydrocortisone. Dehydrogenation of DOC at C-1 and C-2 did not change the Na-retaining property of this compound from that of the parent steroid. 1-dehydro-9α-fluorohydrocortisone is extremely active in retaining Na and preventing symptons of adrenal insufficiency at low dosage and in these respects ranks with aldosterone. It induced a sustained elevation in mean blood pressure and serum Na at dose levels of 25–50 μg./day.

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PROLACTIN AS A FACTOR IN THE VENTRAL PROSTATE ASSAY FOR LUTEINIZING HORMONE

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THE ventral prostates of hypophysectomized castrate rats treated with androgen are somewhat smaller than those of similar animals which are merely castrated. It has been found that this difference can be eliminated if the hypophysectomized castrates are treated with prolactin (1). We have observed that crude alkaline pituitary extracts and many preparations of growth hormone also have this property, but recent studies have led us to attribute it to the prolactin contained in these preparations.

These observations suggest that the reported separation of follicle stimulating hormone and luteinizing hormone activity of gonadotrophins prepared from human non-pregnancy urine may indeed be an artefact (2). We believe that what actually happens is separation of the gonadotrophic hormone from prolactin contamination. We have extensively reviewed the evidence supporting this view and have utilized it to fortify our belief that the human urinary gonadotrophin is a single molecular entity with follicle stimulating hormone and luteinizing hormone activity but attached to various carrier proteins. This view is gradually receiving more and more support (3).

It is the object of this paper to present the data which would indicate that prolactin is indeed a factor in the assay of luteinizing hormone by the hypophysectomized immature rat ventral prostate method (4) and that prolactin from several species of animals displays this synergistic property.

At present sufficiently purified preparations of either human urinary or hypophyseal prolactin are not available for adequate testing in this manner. However, attempts are now being made to prepare such material.

MATERIAL AND METHODS

All studies were carried out on immature Sprague-Dawley male rats, either hypophysectomized or hypophysectomized and castrated in our laboratory (AOMF) at a weight of 75 to 85 grams. In the majority of instances therapy was started seven days after operation, but in some it was begun after only two days, and in others after two weeks.

In all instances the separate materials were injected twice daily for four days as

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different solutions at different sites in the animal. The rats were killed and the ventral prostates and accessary organs were dissected out under a binocular dissecting microscope and weighed immediately on a Roller-Smith torsion balance. The few incompletely hypophysectomized animals were not employed in the statistical or other evaluations of the results.

It would seem appropriate here to consider for a moment the preparations of prolactin employed. All except one were prepared by one of us (S.L.S.) and varied greatly in potency. The one exception to this was a sheep prolactin preparation[1] which has 35 I.U. of prolactin per milligram, the most active preparation used. The least active preparations employed were those of porcine origin. It is noteworthy that we have never seen a prolactin preparation of porcine origin which produced a good dosage-response curve. By the pigeon crop method, a plateau is reached following an early increase in response with increase of dosage. The preparation employed in the present study was assayed on the basis of its potency during the increasing portion of the curve.

In all studies the Armour reference standard Luteinizing Hormone, which is of ovine origin, was employed (SL-227–80). It was prepared by one of us (S.L.S.). In all studies the daily dosage was either 120 or 240 μg. These amounts were almost always adequate to produce a significant increase in the ventral prostate weight.

RESULTS

Results in a typical experiment are shown in Figure 1. Here we see that the L.H. preparation alone is capable of increasing the weight of the ventral

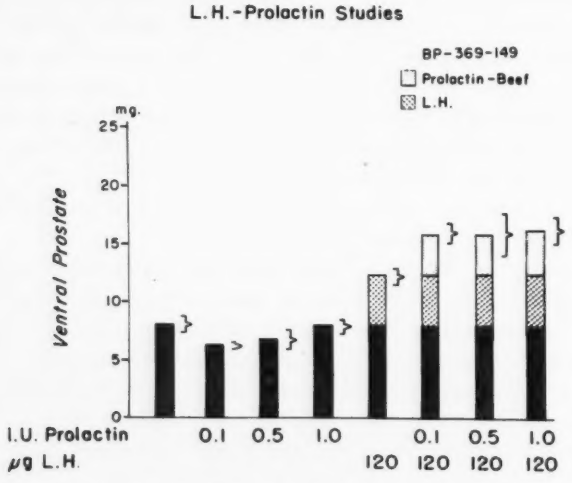

FIG. 1. Effect of beef prolactin and sheep luteinizing hormone on ventral prostate weight.

prostate by 50% over that of the saline treated controls. It is of further interest to note here, as in the subsequent graphs, that administration of prolactin alone usually produces a decrease in the weight of the ventral prostate. However, when the two agents were administered together, there

[1] This sheep prolactin preparation was generously supplied to us by Dr. C. H. Li of the University of California, Berkeley, California.

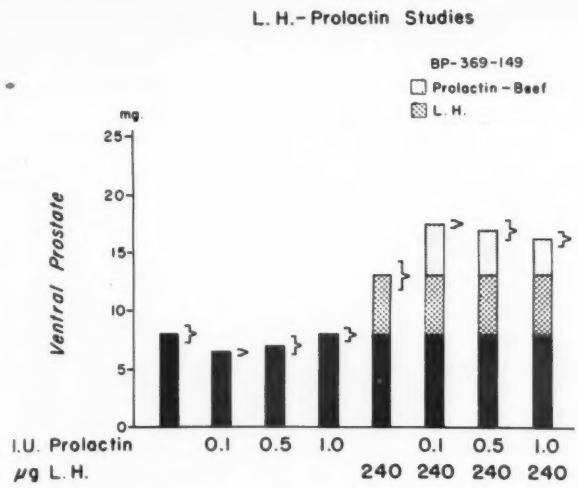

FIG. 2. Effect of beef prolactin and sheep luteinizing hormone on ventral prostate weight.

was twice the increment in ventral prostate weight as seen in the L.H. alone. There does not appear to be any dosage effect for the prolactin, but it appears to be an "all or none" phenomenon. Indeed, the increase produced with 0.1 I.U. daily is as great as that with 1.0 I.U. daily.

Figure 2 shows results when the first experiment was duplicated except that the larger amount (240 μg./day) of L.H. was employed. It is interesting that the increase in ventral prostate weight is the same as that produced

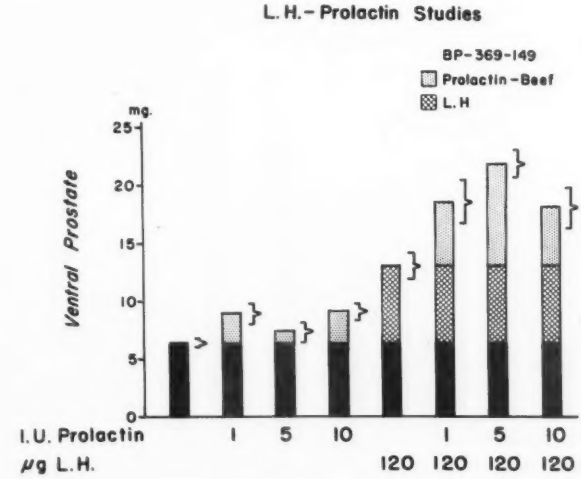

FIG. 3. Effect of beef prolactin and sheep luteinizing hormone on ventral prostate weight.

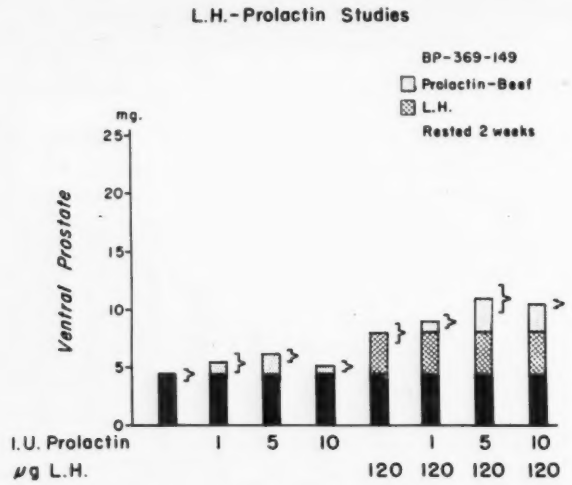

FIG. 4. Effect of beef prolactin and sheep luteinizing hormone on ventral prostate weight. Animals rested two weeks after hypophysectomy.

with the smaller amount of L.H., and the effects from the prolactin, both alone and in combination with L.H., are almost exactly the same.

Figure 3 shows results of a similar experiment. In this study the prolactin produced an increase in the ventral prostate weight when administered alone; however, this is of lesser magnitude than that produced by the standard 120 μg. of L.H. Nonetheless, there was a greater than additive increase in the ventral prostate weight when both preparations were given together.

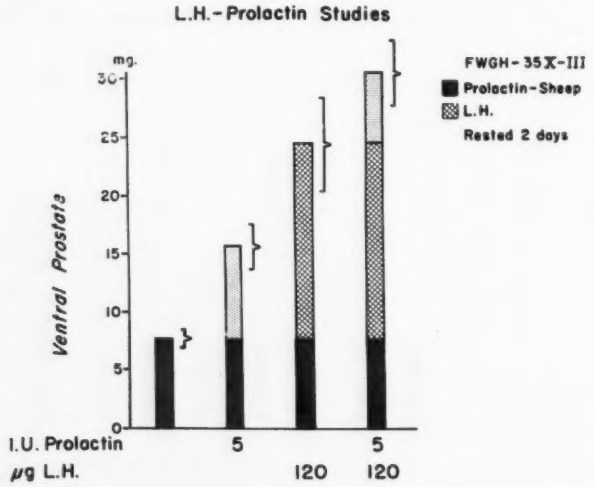

FIG. 5. Effect of sheep prolactin and sheep luteinizing hormone on ventral prostate weight. Animals rested only two days after hypophysectomy.

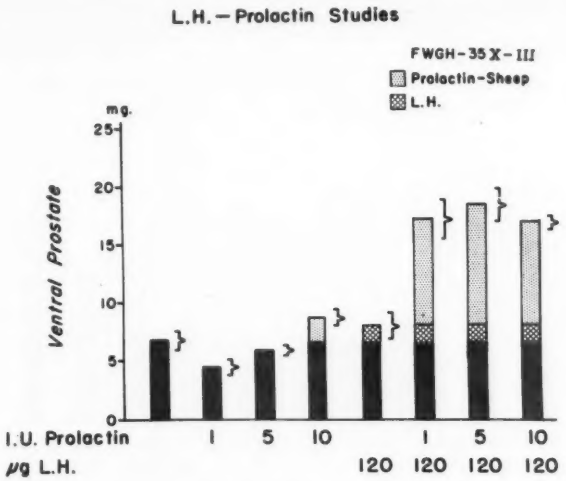

FIG. 6. Effect of sheep prolactin and sheep luteinizing hormone on ventral prostate weight.

Figure 4 shows the effect of beef prolactin when the animals were permitted to rest for two weeks after hypophysectomy. The smaller size of the control ventral prostates should be noted and also the lesser response to the L.H. and to the prolactin. All responses are of small magnitude, but the additional increment obtained with 5 and 10 I.U. of prolactin is significant with P values less than 0.05.

Figure 5 shows results with sheep prolactin, but the graph was designed

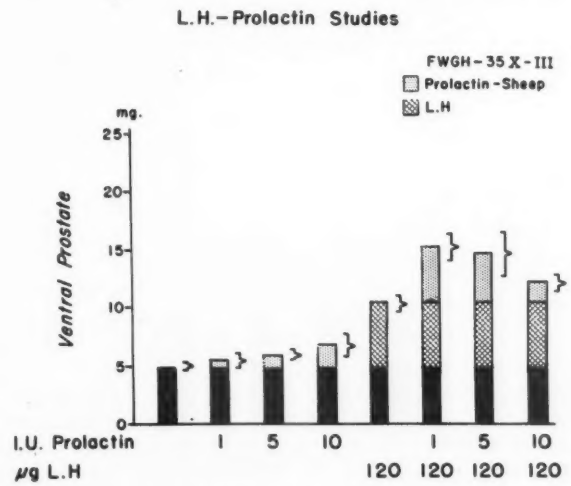

FIG. 7. Effect of sheep prolactin and sheep luteinizing hormone on ventral prostate weight.

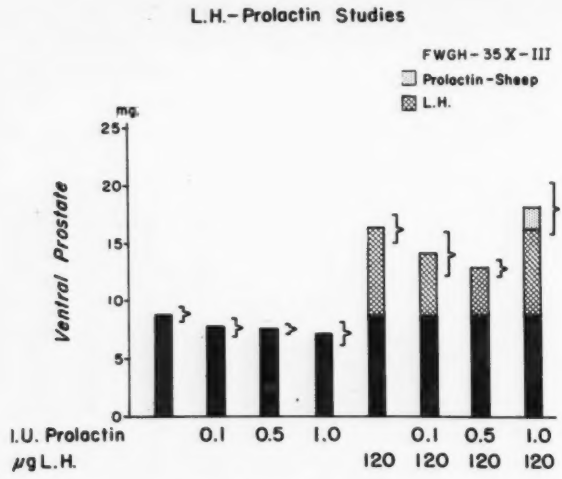

FIG. 8. Effect of sheep prolactin and sheep luteinizing hormone on ventral prostate weight.

to show the converse of Figure 4; here the interval between operation and initiation of therapy was only two days. The great initial weight of the prostate, the tremendous effect from the prolactin alone, the substantial effect of the L.H. and the less than additive effect of the combination can be clearly seen. It is our belief that this represents a carry-over effect of the animal's own pituitary so soon after its removal.

Figure 6 shows the small effect from 120 μg. of L.H. and an equivalent

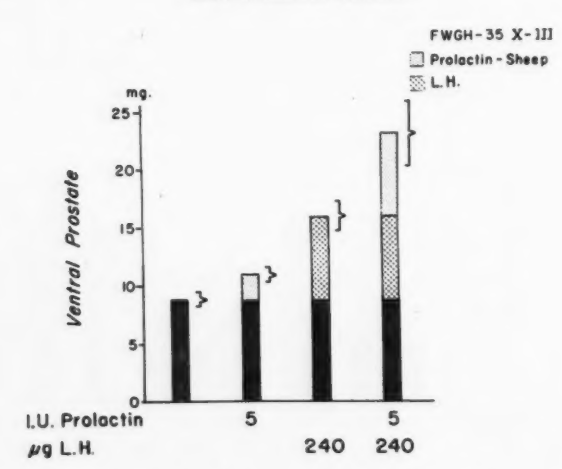

FIG. 9. Effect of sheep prolactin and sheep luteinizing hormone on ventral prostate weight.

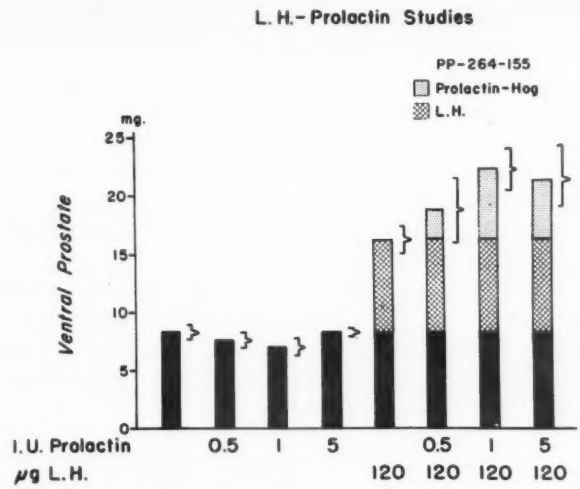

FIG. 10. Effect of hog prolactin and sheep luteinizing hormone on ventral prostate.

increase in the weight of the ventral prostate from the 10 I.U. of sheep prolactin. Despite the small response to the L.H. there was a substantial additional increment when the two were given together.

Figure 7 shows results of another experiment in which sheep prolactin was used. The decrease in the increment obtained from the largest (10 I.U.) amount of prolactin is noteworthy.

Figure 8 shows the results of another attempt to establish the minimum

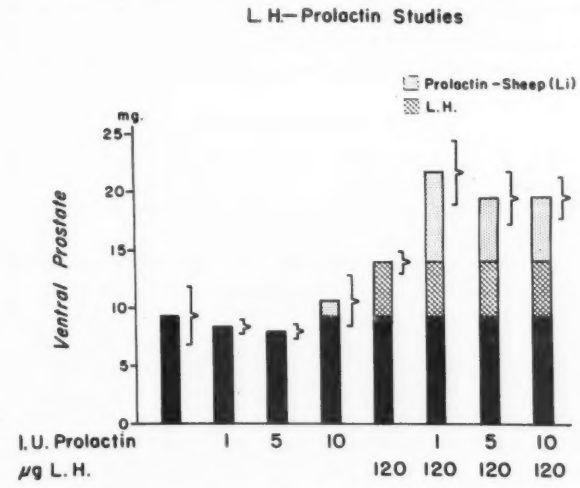

FIG. 11. Effect of sheep prolactin (obtained from Dr. C. H. Li) and sheep luteinizing hormone on ventral prostate weight.

effective dosage of prolactin, this time with a preparation from sheep. The 0.1 and 0.5 I.U. amounts of prolactin actually inhibited the response to L.H. If this graph is compared with Figure 7, it would appear that for this sheep prolactin preparation from 1 to 5 I.U. produces the optimal sensitization of the ventral prostate to the action of the L.H. induced androgen.

Figure 9 serves to illustrate that sheep prolactin also synergizes the action of 240 micrograms of L.H.

Figure 10 shows the typical synergistic effect, but here a preparation of prolactin of porcine origin was employed.

Figure 11 shows the effect from the highly purified sheep prolactin preparation obtained from Dr. Li. A glance at the graph indicates that this too shows the typical effect of syngergism. It also shows a characteristic of the other sheep preparations, namely, that the weight of the ventral prostate increased with 10 I.U. of prolactin alone.

DISCUSSION

In general, the seminal vesicle weights showed changes similar to those seen in the ventral prostate, but the results were neither as consistent nor as striking as those of the ventral prostate.

Weight of the testis was occasionally increased, the greatest increase being produced by the combination, but no significant consistent effects on weight or on microscopic appearance were seen.

The studies which were done two days after hypophysectomy suggest that there is still some carry over of the prolactin effect from the animal's own pituitary.

SUMMARY

It has been shown that prolactins obtained from hogs, sheep and cows sensitize the hypophysectomized rat ventral prostate to the action of androgen produced by the administration of luteinizing hormone.

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EFFECTS OF GROWTH HORMONE AND NUTRITIONAL STATUS ON CARDIAC GLYCOGEN IN THE RAT[1]

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THE maintenance or increase of muscle glycogen stores under the influence of the anterior pituitary gland, its extract (APE), or purified growth hormone has been observed by a number of investigators. In the course of more recent studies of this effect, Illingworth and Russell (1) showed that growth hormone increased the amount of glycogen in the gastrocnemius, heart and diaphragm of fasting normal rats, and restored to normal the glycogen levels in the corresponding tissues of fasting, hypophysectomized animals; of the three types of muscle studied for glycogen storage, the heart was found to be affected to the greatest extent by growth hormone. Russell and Bloom (2) noted the contrast between normal and hypophysectomized rats in the effect of fasting on cardiac glycogen, an increase occurring in the former and a decrease in the latter, and found also that administration of growth hormone to hypophysectomized rats during the fasting period permitted the occurrence of the normal increase in glycogen. Only rather large amounts of growth hormone were used in these investigations. The present studies were prompted by the need for more systematic studies relating varying doses of growth hormone to muscle glycogen levels under diverse experimental conditions.

A preliminary communication about part of the material discussed in this paper has been presented earlier (3).

MATERIAL AND METHODS

In all experiments, male rats of the Sprague-Dawley strain were used. For the establishment of the dose-response relationships, observations were made both in hypophysectomized and in normal animals. The hypophysectomized animals, operated not less than one month prior to the date of the experiment, were 2.5 to 3.5 months old, and weighed 150 to 200 gm. The intact animals were 2.5 to 4 months old, and weighed 200 to 400 gm. The animals were fed Purina Laboratory Chow *ad libitum*, and were main-

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[2] Baxter Laboratories Fellow, 1952–53.

tained at 24–26° C, in groups of two, three or four per cage. In the experiments concerning the refeeding of different foodstuffs, the following quantities of approximately equivalent calorific value were administered: for carbohydrate, 2 gm. of corn starch in aqueous suspension; for fat, 1 ml. of olive oil made into an aqueous emulsion with small quantities of glyceryl monostearate C (Colgate-Palmolive-Peet), oleic acid and sodium taurocholate as adjuvants; for protein, 1.5 gm. of "pure" casein (Coleman and Bell). The carbohydrate and fat were fed by stomach tube; the protein was offered in the form of soft pellets. Fasting, where required, was started in the morning hours, between 8:00 and 9:00 o'clock, in order to eliminate possible effects of diurnal variations.

All but one of the several batches of growth hormone employed were prepared in this laboratory by the method of Wilhelmi, Fishman and Russell (4); the preparations were electrophoretically homogeneous, and contained a low order of contamination. The sample obtained from the Armour Laboratories (Lot No. 22KR2) was also prepared by the same method; it contained some thyrotrophic hormone, but little adrenocorticotrophin. The hormone was administered intraperitoneally, dissolved in physiological saline and rendered slightly alkaline with NaOH in the preparation of the stronger solutions. The total dose of the hormone was contained in a volume of 0.45 to 0.60 ml. The solutions were prepared fresh on the morning of the day on which they were used, and were preserved in the cold when not in use. In the case of the hypophysectomized animals, the total dose of hormone was administered in three approximately equal portions, at 9:30 A.M., 1:30 to 2:30 P.M. and 10:00 to 11:00 P.M. In other cases, it was given as indicated for the various experiments.

In the hypophysectomized animals, the responses were measured in terms of trichloroacetic acid-extractable (TCA-extractable) glycogen, on the assumption that this more labile fraction (5) would also be more sensitive to the effects of the hormone. In all other experiments, both TCA-extractable and "residual" (*vide infra*) fractions were determined. For the removal of tissue, established techniques (5, 6, 7) were employed in order to reduce the possibility of loss of glycogen by enzymatic breakdown. The order of excision of tissues was as follows: gastrocnemius muscle, heart, liver. The isolation and determination of the two glycogen fractions in the tissues was carried out according to the modified technique adopted by Russell and Bloom (2, 5) on the basis of the earlier method described by Bloom *et al.* (8), with one important point of difference in procedure. Whereas in the papers cited residual glycogen was determined by difference, in the present experiments both fractions were determined on the same sample. After the clear trichloroacetic acid extract was drained off the centrifuge tube, the glycogen in the residual tissue was isolated by the Pfluger method, leading to the estimation of the "residual" fraction. The water content of the frozen tissues, representing various stages of fasting and hormonal treatment, was fairly uniform, as indicated by the following analysis; 27 samples, dried to constant weight at 110° C, showed a water content of 75.7–77.6%.

EXPERIMENTAL DESIGN AND RESULTS

1. *Growth hormone treatment of hypophysectomized animals:* Hypophysectomized animals were treated during a 24-hour fasting period with varying quantities of growth hormone, and the TCA-extractable glycogen of gastrocnemius muscle and of the heart were determined at the end of this time. The results are summarized in Table 1. Series I and II of the experiments were performed with the same batch of hormone but some months apart, and some degree of deterioration of the hormone may have occurred during this time. For Series III, Armour's preparation was used. In all cases, the

TABLE 1. EFFECT OF GROWTH HORMONE ON TRICHLOROACETIC ACID-EXTRACTABLE MUSCLE GLYCOGEN IN HYPOPHYSECTOMIZED RATS*

Type of animal and treatment	Gastrocnemius muscle			Cardiac muscle		
	No. of observ.	Glycogen mg.%	Slope and λ	No. of observ.	Glycogen, mg.%	Slope and λ
Normal untreated	14	230 ± 10†		12	354 ± 8†	
Hypophysectomized untreated	11′	144 ± 8		9′	215 ± 18	
	7″	165 ± 11		7″	169 ± 13	
Hypophysect. + G.H. (μg./100 gm.)						
Series I‡						
31.25	4	153 ± 12	Slope =	4	255 ± 10	Slope =
62.5	11	179 ± 7	50 ± 7	6	238 ± 11	116 ± 21
125	11	192 ± 7		9	279 ± 17	
250	10	198 ± 10	λ =	10	350 ± 20	λ =
500	11	226 ± 6	0.476	9	358 ± 15	0.433
1000	7	232 ± 8		6	361m ± 12	
Series II‡						
62.5	4	166 ± 27	Slope =	3	259 ± 11	Slope =
125	4	194 ± 10	79 ± 25	4	233 ± 28	52 ± 32
250	6	243 ± 14	λ =	6	277 ± 20	λ =
500	6	233*m* ± 22	0.475	6	290 ± 16	0.834
Series III‡						
62.5	4	209 ± 22	Slope =	4	284 ± 12	Slope =
125	4	241 ± 12	40 ± 37	4	364 ± 21	80 ± 48
250	4	233 ± 10	λ =	4	332 ± 11	λ =
500	4	246 ± 10	0.768	4	315m ± 19	0.513
Composite			Slope = 54 ± 15 λ = 0.525			Slope = 98 ± 27 λ = 0.480

* Fasted 24 hours; G.H. administered in three approximately equal portions during the fasting period.
† Standard error.
‡ See text for G.H. preparations used in each series.
′ Controls for Series I.
″ Controls for Series II and III.
m—See text for explanation.

effective dose of the hormone appeared to lie within the range of 62.5 to 500 μg./100 gm., the latter dosage inducing maximal effects. The glycogen levels marked "m" in the tables are not significantly higher than those of the preceding lower doses, and were eliminated before calculation of statistical figures. The dose response relationships were significant and linear in most cases. The slopes of the various series did not differ significantly, but the indices of precision were rather high.

2. *Cardiac glycogen in intact animals:*

Time course of the effect of fasting on cardiac glycogen levels. Before the possible effects of growth hormone in intact rats could be investigated, it was necessary to define further the effects of nutritional status on the glycogen of the heart. The alterations in cardiac glycogen during periods of fasting ranging from 6 hours to 7 days are presented in Figure 1. Here,

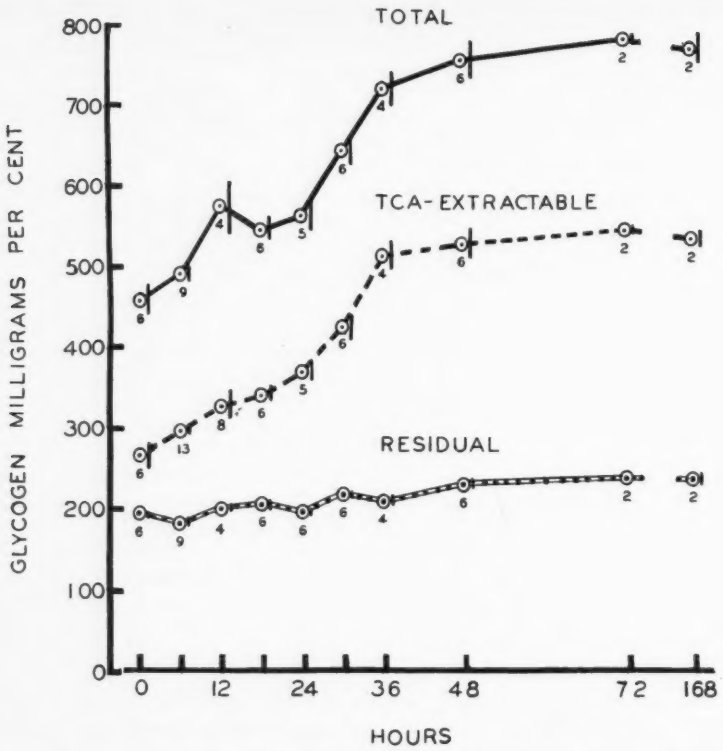

FIG. 1. Time course of the effect of fasting on cardiac glycogen in normal rats. The small numerals indicate the number of observations for each point; the vertical lines show the standard errors.

fasting had induced a progressive rise in total glycogen from the unfasted level of 450 mg.% to a plateau of 770 mg.%, attained within 48 hours, and maintained during a fasting period of 7 days. This rise was to a very great extent reflected in the TCA-extractable fraction, the residual fraction exhibiting comparatively little change.

Effect of refeeding different foodstuffs on cardiac glycogen levels. In order to determine whether any particular type of foodstuff was responsible for the repressive effect of feeding in cardiac glycogen, groups of animals were fasted during 48 hours and then fed carbohydrate, protein or fat in quantities of equivalent calorific value. In some experiments the amount of fat was doubled, with no apparent difference in results. For each foodstuff, analyses of cardiac glycogen were made at 6 hours after the feeding (54th hour from the beginning of fasting), and also 12 hours later (60th hour); in the latter case food was given a second time, at the 54th hour. The results are presented in Table 2, together with the total glycogen levels of the liver, determined at the same time. Feeding of either carbohydrate or

TABLE 2. EFFECT OF REFEEDING DIFFERENT FOODSTUFFS ON CARDIAC AND HEPATIC GLYCOGEN IN RATS

Nutritional state	No. of observ.	Heart glycogen, mg.%			Liver glycogen, mg.%
		TCA-extract	Residual	Total	Total
Unfasted	6	265 ± 29*	192 ± 5	457 ± 33	
Fasted 48 hours	6	527 ± 38	230 ± 11	757 ± 44	
Carbohydrate fed†					
6 hours	3	357 ± 10	221 ± 3	578 ± 8	4354 ± 342
12 hours	3	284 ± 24	168 ± 11	452 ± 35	6532 ± 926
Protein fed†					
6 hours	4	442 ± 42	202 ± 9	644 ± 47	2402 ± 236
12 hours	3	344 ± 43	192 ± 12	536 ± 36	3729 ± 453
Fat fed†					
6 hours	5	465 ± 16	257 ± 10	722 ± 38	959 ± 83
12 hours	6	498 ± 20	268 ± 8	766 ± 31	393 ± 50

* Standard error.
† Fed after 48 hours of fasting.

protein was followed by depression of cardiac glycogen levels. In the case of carbohydrate, the normal, unfasted level was regained within 12 hours. With protein, a slightly higher level was observed at this time, but the decrease from fasting was still quite significant. The effect of fat was quite different: after an insignificant fall at the 54th hour, the normal fasting level was maintained to the 60th hour. Hepatic glycogen in animals fed carbohydrate or protein was high, whereas in the fat fed animals values ranged approximately from 0.4 to 1%, the lower values being near those seen in fasting animals.

Effect of growth hormone on cardiac glycogen in intact animals: Before the responses to graded doses of the hormone were determined, an attempt was made to find the conditions under which the greatest responses could be elicited by a given dose of hormone. The time during which the hormone was allowed to exert its effect and the stage of fasting were two important factors.

a. The effect of a single dose of 500 μg. of growth hormone allowed different periods of activity is indicated in Table 3. The cardiac glycogen

TABLE 3. EFFECT OF GROWTH HORMONE ON CARDIAC GLYCOGEN: SINGLE DOSE OF 500 μg. ALLOWED DIFFERENT PERIODS OF ACTIVITY

Hours	Glycogen levels in G.H. treated animals						Increase in glycogen over untreated controls					
	TCA-ext. glycogen		Residual glycogen		Total glycogen		TCA-ext. glycogen		Residual glycogen		Total glycogen	
	N*	mg.%	N	mg.%	N	mg.%	D.F.†	mg.%	D.F.	mg.%	D.F.	mg.%
6	14	419 ± 32	14	216 ± 12	14	635 ± 35	25	123 ± 35	21	36 ± 16	21	144 ± 46
12	7	602 ± 66	7	213 ± 14	7	815 ± 78	13	277 ± 73	9	14 ± 20	9	240 ± 115
18	7	459 ± 17	7	199 ± 10	7	658 ± 13	11	120 ± 25	11	−6 ± 15	11	114 ± 28
24	6	540 ± 50	6	196 ± 14	6	736 ± 63	9	172 ± 61	10	0 ± 16	9	173 ± 77

* Number of observations.
† Degrees of freedom $= n_1 + n_2 - 2$.

values from animals given a single dose of growth hormone at the onset of the fast and sacrificed 6 to 24 hours later are listed along with the increments in glycogen over untreated fasting levels. In all instances, growth hormone accelerated the attainment of fasting levels, or more correctly, allowed the attainment of levels even higher than those induced by fasting in the same period of time. The increment over corresponding untreated levels was perhaps greatest at the 12th hour.

b. The effect of a dose of 500 μg., repeated every 6 hours during intervals ranging from 6 to 24 hours, is given in Table 4. The first dose was adminis-

TABLE 4. EFFECT OF GROWTH HORMONE ON CARDIAC GLYCOGEN: 500 μg. DOSE REPEATED EVERY 6 HOURS DURING DIFFERENT PERIODS

Hours from first dose	Glycogen levels in G.H. treated animals						Increase in glycogen over untreated controls					
	TCA-ext. glycogen		Residual glycogen		Total glycogen		TCA-ext. glycogen		Residual glycogen		Total glycogen	
	N*	mg.%	N	mg.%	N	mg.%	D.F.*	mg.%	D.F.	mg.%	D.F.	mg.%
12	6	815 ± 47	5	215 ± 16	4	1084 ± 52	12	490 ± 58	7	16 ± 20	6	509 ± 84
18	6	630 ± 37	5	213 ± 18	5	844 ± 62	10	291 ± 31	9	8 ± 19	9	300 ± 63
24	7	761 ± 30	7	218 ± 15	7	979 ± 33	10	393 ± 42	11	22 ± 18	10	416 ± 49

* See footnotes of Table 3.

tered at the beginning of the fasting period, and the last was given 6 hours before the animal was sacrificed. The increase in glycogen appeared to reach its maximum after two doses of hormone, and to remain elevated at later hours.

c. In view of the preceding results, a few preliminary comparisons were made of responses to graded doses of hormone (31.25 to 2000 μg.) under three different conditions: (1) single dose allowed 6 hours; (2) the same dose allowed 12 hours; (3) the same total dose given in halves, one at the beginning and the other at the middle of a 12-hour fasting period. Since neither of the 12-hour methods appeared to be greatly superior to the shorter and simpler method of the 6-hour interval, further data on the dose-response relationships were obtained by the latter procedure. The results are presented in Table 5. The minimum effective dose of the hormone was about 30 μg./100 gm., with the response increasing gradually to a maximum at the dose of 2000 μg. The parameters of the dose-response curve, shown at the bottom of the same table, were calculated after some modification of the data. A few highly aberrant values or "outliers," that did not seem to belong to the particular group in which they occurred, were eliminated after being tested by Dixon's method and criteria (9). The responses to the 2000 μg. dose also were omitted, because they were inordinately high compared with those for the lower doses. That the deviation from linearity introduced by this dose could not be attributed to chance was concluded on the basis of an analysis of variance for linearity: F values of 6.3 and 7.9 obtained for TCA-extractable and total glycogen respectively

corresponded to p values of less than 0.01 in both fractions. With the data as modified, the relationship between doses and responses appears to be linear and highly significant, and even the smallest dose of hormone is seen to have brought about a significant rise in total and TCA-extractable glycogen. In the case of residual glycogen the slope, although significant, is very low. The indices of precision for total and TCA-extractable glycogen both are somewhat higher than the composite index for TCA-extractable cardiac glycogen obtained in the case of hypophysectomized animals; with residual glycogen the index is even higher.

TABLE 5. EFFECT OF GROWTH HORMONE ON CARDIAC GLYCOGEN: SINGLE DOSES ALLOWED 6 HOURS OF ACTIVITY

Treatment	TCA-ext. glycogen		Residual glycogen		Total glycogen	
	N*	mg.%	N	mg.%	N	mg.%
Untreated controls	13	296 ± 10	9	180 ± 9	9	491 ± 17
G.H.						
31.25 μg.	7	359 ± 17	7	208 ± 11	7	567 ± 14
62.5 μg.	8	413 ± 40	8	229 ± 9	8	642 ± 42
125 μg.	6	367 ± 29	6	237 ± 9	6	603 ± 22
250 μg.	9	412 ± 21	9	249 ± 5	9	662 ± 24
500 μg.	6	460 ± 67	6	247 ± 14	6	706 ± 65
1000 μg.	6	508 ± 50	6	255 ± 11	6	762 ± 57
2000 μg.	6	750 ± 89	6	317 ± 13	6	1067 ± 99
Slope		103 ± 29		28 ± 9		133 ± 29
Index of precision (λ)		0.866		0.905		0.687
Significance of linearity (F)		19		12.7		12.2

* Number of observations.

The combined effect of growth hormone and feeding in intact animals. In an earlier experiment (Table 2) it was seen that the high cardiac glycogen values attained in fasting were rather abruptly restored to normal levels by the feeding of carbohydrate or protein. Such an effect might be interpreted as resulting either from a decrease in secretion of a pituitary factor or from inhibition of the activity of circulating hormone. To test the latter possibility, the effect of simultaneous growth hormone administration and feeding was observed in the following way. The animals were first fasted overnight, and then allowed free access to their usual food. Graded doses of growth hormone were then administered, one half at the start of the refeeding period and the other half 6 hours later, and the cardiac glycogen determined 12 hours after the food was first given. For comparison, another series of animals were subjected to similar hormonal treatment during a 12-hour period of continued fasting. The results are presented in Table 6.

It will be seen that with either the 250 μg. or 500 μg. dose the increment in total glycogen over the corresponding control level in the fed animal was not significantly different from that induced in the fasting animal. In the

TABLE 6. COMPARISON OF THE EFFECTS OF GROWTH HORMONE ON ANIMALS IN THE FED AND FASTING STATES*

	Glycogen levels in G.H. treated animals						Increase in glycogen over untreated controls					
	TCA-ext. glycogen		Residual glycogen		Total glycogen		TCA-ext. glycogen		Residual glycogen		Total glycogen	
	N*	mg.%	N	mg.%	N	mg.%	D.F.†	mg.%	D.F.	mg.%	D.F.	mg.%
Fed Animals												
Untreated controls	6	234 ± 13	6	194 ± 10	6	428 ± 14						
G.H.												
250 μg.	4	275 ± 12	4	216 ± 8	4	491 ± 16	8	41 ± 19	8	22 ± 14	8	63 ± 21
500 μg.	7	409 ± 47	7	248 ± 9	7	657 ± 55	11	175 ± 53	11	54 ± 13	11	229 ± 61
1000 μg.	6	398 ± 14	6	263 ± 6	6	661 ± 17	10	164 ± 19	10	69 ± 12	10	233 ± 22
Fasting Animals												
Untreated controls	8	325 ± 36	4	199 ± 10	4	575 ± 66						
G.H.												
250 μg.	6	430 ± 27	6	243 ± 15	6	672 ± 21	12	105 ± 50	8	44 ± 21	8	97 ± 59
500 μg.	5	499 ± 38	5	274 ± 5	5	773 ± 38	11	174 ± 55	7	75 ± 10	7	198 ± 73
1000 μg.	6	815 ± 47	5	218 ± 16	4	1084 ± 52	12	490 ± 54	7	16 ± 20	6	509 ± 84

* See text for the hours of feeding and fasting and mode of administration of hormone.
† See footnotes of Table 3.

case of the 1000 μg. dose, however, the increment in the fasting animal was apparently much higher. It may also be noted that doses of 500 and 100 μg. allowed the attainment of a normal 30-hour fasting level (Fig. 1), overcoming the effects of feeding completely.

DISCUSSION

From the data presented above, it appears that the effects of highly purified growth hormone on muscle or cardiac glycogen storage in hypophysectomized or on cardiac glycogen in intact rats is significantly related to dosage of hormone. As these changes could be produced by relatively small doses (30 to 500 μg. approximately), it is not improbable that the effect observed is physiological. The log dose-response relationships were approximately linear, and where preparations differing in potency have been "assayed" on the basis of their effects on gastrocnemius and cardiac glycogen in hypophysectomized rats, the regression lines for these preparations were essentially parallel. The composite indices of precision (λ values) obtained for the gastrocnemius (0.525) and for the heart (0.480) in hypophysectomized animals lie within the range of values (0.198–0.670) published for established methods of growth hormone assay (10); it is apparent, however, that our values approach the higher limits of the range. In the case of intact animals, the indices of precision are somewhat higher, being 0.687 for total glycogen and 0.866 for the TCA-extractable fraction. The higher index for TCA-extractable glycogen does not support the supposition, mentioned earlier, that assay methods based on this fraction might be more accurate. Because of the relatively low order of precision, a completely satisfactory procedure for bioassay of the "glycostatic" factor cannot as yet be recommended on the basis of our present data. However,

the method does afford some measure of the activity of pituitary preparations based upon an easily measured and specific metabolic effect. Insofar as the test is specific for growth hormone (see below), it would also allow the rough assay of this factor by a quick and fairly sensitive method.

The progressive rise of cardiac glycogen in fasting rats, described above, extends the data of other investigators (2, 7, 11). According to the findings reported in Table 2, both carbohydrate and, to a somewhat lesser extent, protein caused a depression of glycogen levels, while fat exerted no such effect. Hepatic glycogen levels were very high in the carbohydrate fed animals, somewhat lower but normal in the case of protein feeding, but very low and not significantly different from fasting levels in fat fed animals. These observations may be correlated on the basis of the assumption that the repressive effects of feeding are due essentially to carbohydrate, either as such or in the form of some of its metabolic products. The similar, but less pronounced, effects of protein may be the result of gluconeogenesis. The question remains as to the mechanism of this paradoxical effect of carbohydrate on the cardiac glycogen. Since the glycogen content of the heart does not increase during fasting in the absence of the hypophysis but does so if growth hormone preparations are given, it seems likely that the effect of nutritional state is mediated via a pituitary hormone. In this case it must be assumed that either the secretion or the activity of the hormone is augmented in the fasting or fat fed animal, and that this effect is repressed by feeding carbohydrate or its precursors.

If growth hormone is the factor responsible for the changes in cardiac glycogen, then it should be possible to simulate the effects of fasting by means of exogenous growth hormone in the intact animal, and as shown above, this expectation has been verified. A single dose of growth hormone accelerated the attainment of fasting levels of cardiac glycogen or allowed the attainment of levels even higher than those induced by fasting alone, and with repeated administration of hormone, still greater increments in glycogen levels were obtained (Table 4). The latter effect could have been due either to the continued presence of hormone concentrations approximating those obtained with the single dose, or to a cumulative action tantamount to the effect of a larger single dose of hormone.

In an attempt to find out whether the nutritional state affected the activity of the pituitary factor on cardiac glycogen, growth hormone was given along with food to animals previously fasted 12 hours (Table 6). Since the difference in glycogen content between the hearts of the control and of hormone treated animals was similar to that seen in fasting rats given the hormone alone, and since the hormone completely prevented the expected effects of feeding, the evidence obtained does not support the view that feeding interferes with or inhibits the activity of the hormone on cardiac glycogen. These experiments may not be entirely conclusive, however, for in some other exploratory trials little effect on the glycogen of the heart

was seen after the administration of growth hormone over 24 hours to fully fed rats.

The question may be raised whether the effects on tissue glycogen described above are produced by growth hormone itself or by some other pituitary factor. All of the many highly purified bovine growth hormone preparations which have been tested have been found to maintain the gastrocnemius glycogen levels in fasting hypophysectomized rats, and the order of activity has been similar in all cases. With a smaller number of preparations, similar observations have been made in respect to their ability to bring about augmentation of the cardiac glycogen in hypophysectomized or intact rats. Purified adrenocorticotrophic hormone is not active in the maintenance of the gastrocnemius glycogen in the hypophysectomized rat during a 24-hour fast, nor is the adrenal cortex required for the activity of other pituitary preparations in this test (12, 13). Hence, it appears that this action is the property either of growth hormone or of an unidentified factor which remains closely associated with growth hormone in its preparation. With respect to the factor which augments or allows the increase in cardiac glycogen, the relationship to adrenocortical activity is not yet entirely clear. In line with Astwood's reports (14), we have observed that some preparations of ACTH will bring about an increase in the cardiac glycogen in an acute test (6 hours) in intact rats. However, the adrenalectomized rat, unlike the hypophysectomized animal, displays the normal increase in cardiac glycogen during a 24-hour fasting period (2). Since it has been observed earlier (1, 15) that the cortical hormones exert a permissive or synergistic effect in the acute deposition of glycogen in the gastrocnemius of glucose-fed animals, it is not unlikely that the similar acute "glycostatic" effect of the corticotrophic hormone on the cardiac glycogen is mediated through the same mechanism. Further experiments are in progress to elucidate the interrelationships of the activities of these hormones in the process of glycogen deposition.

SUMMARY

Studies relating the dose of purified growth hormone to muscle glycogen levels were carried out in hypophysectomized and in intact rats. In the hypophysectomized animal, doses of hormone ranging from 30 to 500 μg./100 gm., administered during a 24-hour fasting period, induced increasing elevations of the trichloroacetic acid-extractable fraction of gastrocnemius and cardiac glycogen. Similar effects occurred in the intact animal, where doses of hormone ranging from 30 to 1000 μg., administered during a 6 hour fasting period, caused the deposition of increasingly higher concentrations of total and trichloroacetic acid-extractable glycogen in the heart; residual glycogen exhibited relatively little change. In all instances, the log dose-response relationships were significant and linear through most of the range, but the indices of precision were relatively high.

On fasting, the intact rat showed a progressive rise in cardiac glycogen from the unfasted level of 400–450 mg. per cent to a plateau of about 770 mg. per cent, attained within 48 hours, and maintained during an additional fasting period of 5 days.

Feeding of carbohydrate or protein of equivalent calorific value at the 48th hour of fasting induced an aburpt fall in cardiac glycogen, while feeding of fat effected no appreciable change.

Exogenous growth hormone, administered in various doses and during varying periods of time to intact fasting rats, accelerated the attainment of levels of cardiac glycogen which were even higher than those induced by fasting alone.

When growth hormone was administered to intact animals along with food at the end of a 12-hour fasting period, its effect was found to be similar to that seen in fasting animals.

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NOTES AND COMMENTS

ENZYMATIC CONVERSION OF THYROXINE AND TRIIODOTHYRONINE TO THE CORRESPONDING ACETIC ACID ANALOGUES[1]

Pitt-Rivers (1) found the acetic acid analogues of thyroxine and triiodothyronine to possess a "high degree of activity" in goiter prevention assay and triiodothyroacetic acid to have considerable activity in increasing the metabolic rate of rats. Interest in these compounds has been heightened by the reports that these acetic acid analogues, unlike the parent compounds, produce an immediate increase in the oxygen consumption of rat kidney slices (2) and a rapid clinical effect in myxedematous patients (3, 4). These observations prompted Thibault and Pitt-Rivers (2) to speculate that these derivatives may be the forms in which the hormones act in the tissues. Roche, Michel and coworkers (5) have reported the occurrence of triiodothyroacetic acid in the kidney of rats given triiodothyronine. The present communication reports the conversion of thyroxine and triiodothyronine to their respective acetic acid analogues *in vitro* by an enzyme prepared from rat kidney mitochondria.

A soluble enzyme system was prepared by dialysis of the supernatant fraction of sonic disintegrated rat kidney mitochondria. The substrates, thyroxine and triiodothyronine, were incubated with this enzyme at 37° C for three hours in the presence of adenosinetriphosphate, coenzyme I, and phosphate buffer (pH 7.4). Following incubation, the reaction mixtures were extracted with 2% ammonium hydroxide in butanol. The butanol extracts were analyzed by paper chromatography.

In the thyroxine experiments three labeled reaction products uniformly appeared (Fig. 1). The extent of conversion of thyroxine to the products increased linearly with time. The principal product (T_4U_1) was eluted from the paper and rechromatographed. The compound gave positive diazo and Kendall tests and negative ninhydrin and 2,4-dinitrophenylhydrazine reactions. The chromatographic mobility of T_4U_1 was compared with several known iodinated thyronine compounds in three different solvent systems (tertiary amyl alcohol-2 N NH_4OH, collidine-water-ammonia and butanol-1 N HCl). In each system it had a mobility identical with tetraiodothyroacetic acid.[2]

In the triiodothyronine experiments two labeled reaction products appeared (Fig. 2). The principal product (T_3U_1) was studied as above. This compound also gave positive diazo and Kendall reactions and negative ninhydrin and 2,4-dinitrophenylhydrazine reactions. The chromatographic mobility of T_3U_1 in the three solvent systems was identical with triiodothyroacetic acid.[2] Further identification of the T_3U_1 was accomplished by the finding of a constant specific activity in five successive recrystallization experiments using synthetic triiodothyroacetic acid.

The propionic acid analogues were found to have similar, but not identical, mobility in the solvent systems used. The biosynthetic acetic acid analogues were easily differ-

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[2] Obtained through the courtesy of Dr. Jack Gross, State University of New York College of Medicine, Brooklyn, New York.

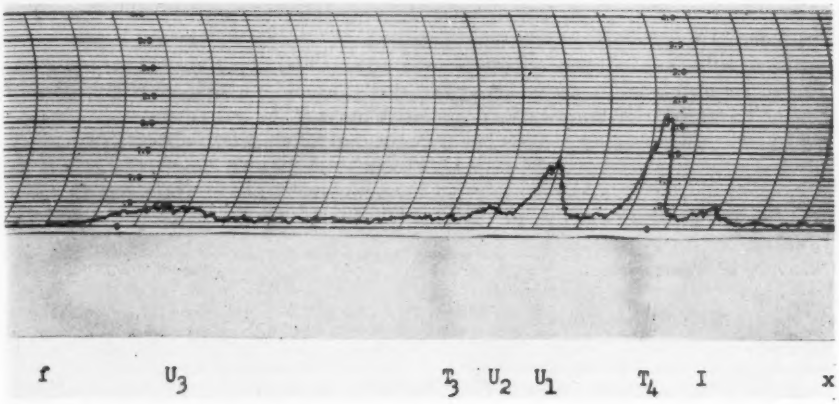

FIG. 1. Chromatographic pattern of butanol-ammonia extract of the reaction mixture containing mitochondrial enzyme system with I^{131} labeled thyroxine as substrate. Tertiary amyl alcohol-2 N NH_4OH solvent system.

X—origin of chromatogram
f—chromatographic front
I—radioactivity as iodide
T_4—thyroxine radioactivity and corresponding carrier thyroxine color band (diazo reaction)
T_3—carrier triiodothyronine color band (diazo reaction)
U_1—radioactivity of principal reaction product, tetraiodothyroacetic acid
U_2U_3—radioactivity of two additional unidentified reaction products

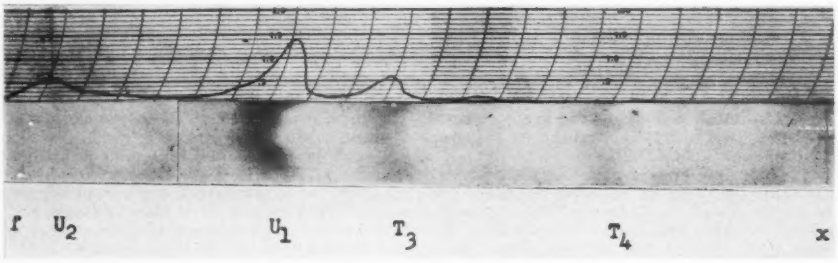

FIG. 2. Chromatographic pattern of butanol-ammonia extract of the reaction mixture containing mitrochondrial enzyme system with I^{131} labeled triiodothyronine as substrate. Tertiary amyl alcohol-2 N NH_4OH solvent system.

X—origin of chromatogram
f—chromatographic front
T_4—carrier thyroxine color band (diazo reaction)
T_3—triiodothyronine radioactivity and corresponding carrier triiodothyronine color band (diazo reaction)
U_1—radioactivity of principal reaction product and corresponding carrier triiodothyroacetic acid color band (diazo reaction)
U_2—radioactivity of unidentified reaction product

entiated from the corresponding tri- or tetra-iodopropionic analogues by the determination of specific activity in recrystallization experiments.

It is possible that the transformation described here is of importance in the formation of a specific metabolically active derivative of the thyroid hormone. It is also possible that the transformation represents a degradative process.

The technical assistance of Miss Sue Ames is gratefully acknowledged.

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REDUCTION IN PAROTID AMYLASE AFTER HYPOPHYSECTOMY[1]

Baker and Abrams (1) demonstrated that the parotid gland loses weight after hypophysectomy. Its acinar cells become smaller and contain fewer zymogenic granules. This report shows that amylase is reduced also.

MATERIALS AND METHODS

Young, adult female rats of the Sprague-Dawley strain were used. They were fed a diet consisting of Purina Laboratory Chow supplemented weekly with oranges, greens, and a vitamin concentrate. Subsequent to the operation, each control animal was pair-fed against a hypophysectomized rat. If the experiment lasted for only 7 or 8 days, the control animal was sham-operated by carrying out all of the procedure for hypophysectomy except aspiration of the gland. In all hypophysectomized animals which were sacrificed after 7–8 days and in most of those sacrificed at 39–78 days, completeness of the hypophysectomy was proven by examination of serial microscopic sections of the pituitary region. In the remainder of the hypophysectomized rats, completeness of removal of the gland was established by the presence of a reduction in body weight and by gross examination of the pituitary region. If fragments of the gland were retained, the animal was discarded from the experiment. The animals were given water ad libitum but no food for 24 hours prior to termination of the experiment.

The parotid was excised while the animal was under sodium amytal anesthesia. The gland was weighed and homogenized in a solution of 0.85% NaCl. After filtering through fiber glass, additional saline solution was added so that a final concentration of 1 gm. of gland in 5000 ml. of homogenate was obtained. To 1 ml. of the homogenate was added 2 ml. of 0.2

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TABLE 1. THE EFFECT OF HYPOPHYSECTOMY ON THE AMYLASE ACTIVITY OF THE PAROTID

Treatment	No. of rats	Days posthyp.	Body wt. (gm.)		Mean par. wt. (mg.)	Mean amylase activity		
			Initial	Final		Per .1 mg. par. (mg. maltose[1])	Total (gm. maltose)	Tot. (gm. maltose)/ Body wt. (gm.) ×100
Con.	6	7– 8	173±1[1]	162±4	101.2±3.4	4.13±0.15	4.25±0.44	2.6±0.08
Hyp.	6		171±2	141±10	70.7±6.8	2.41±0.39	1.82±0.39	1.3±0.08
						P<.001	<.01	<.01
Con.	10	39–78	195±5	235±21	126.7±5.5	4.68±0.3	5.98±0.50	2.6±0.23
Hyp.	12		195±4	174±6	82 ±3.7	2.88±0.23	2.40±0.24	1.4±0.05
						P<.001	<.001	<.001

Hyp. =hypophysectomy, Par. =parotid, Con. =control, Wt. =weight.
[1] Standard error of the mean.

M KH_2PO_4-Na_2HPO_4 buffer (pH 7.2) and 5 ml. of 2% starch solution made up in 4% NaCl, he latter two solutions having been warmed previously to 37° C. A similar preparation was nade as a control except that the enzyme was inactivated by placing the test tube containing he homogenate in boiling water before addition of the phosphate buffer and starch solution. Digestion of both preparations was carried out at 37° C. with constant agitation of the tubes.

After 10 minutes, the test tubes were plunged into boiling water in order to stop enzyme ction in the one which contained active homogenate. Protein was precipitated by the ddition of 1 ml. of 5% $ZnSO_4$ and 1 ml. of 0.3 N $Ba(OH)_2$ and followed by centrifugation at 2000 RPM for 3 minutes.

The Shaffer-Somogyi method was employed for determination of the quantity of reducing substances present in the tubes. For this purpose 5 ml.[2] of the supernatant were added to 5 ml. of the Somogyi copper reagent and placed in a bath of boiling water for 15 minutes. After cooling, 2 ml. of 2.5% potassium iodide-potassium oxalate solution and 10 ml. of 1.0 N H_2SO_4 were added and the solution titrated with 0.005 N sodium thiosulfate. The starch substrate carried over in the supernatant served as an indicator. The difference in the amount of thiosulfate used in titration of the supernatant from the tube which contained active homogenate and that which contained inactivated homogenate was considered equivalent to the amount of reducing material released from starch by the parotid amylase. This was expressed in terms of maltose by reference to a standard curve which gave a coefficient indicative of the amount of maltose equivalent to a unit volume of thiosulfate. The significance of the difference between the mean values for control and experimental groups was determined by the use of the Student Fisher t formula.

OBSERVATIONS AND SUMMARY

Seven to 8 days after hypophysectomy the mean weight of the parotid gland was reduced from 101.2± standard error 3.4 mg. for the controls to 70.7±6.8 after hypophysectomy (Table 1). Concurrently, the concentration of amylase was reduced from a mean of 4.13±0.15 mg. maltose/0.1 mg. of parotid to 2.41±0.39 for the control and hypophysectomized groups, respectively. The reduction in total amylase per gland, and in the ratio of total activity to body weight was significant at the 1% level. At 39–78 days after hypophysectomy, similar changes were observed.

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[2] In current work, this volume has been reduced to 1 ml. for non-hypophysectomized animals.

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ISOLATION OF AN APPARENTLY HOMOGENEOUS FOLLICLE STIMULATING HORMONE

During the past year Steelman, Lamont and Baltes (1, 2) have shown that it is possible to prepare pituitary follicle stimulating hormone (FSH) with a greater specific activity than preparations previously reported to have a high degree of purity (3, 4). Peterson and Sober (5) have recently published studies which show that proteins can be fractionated on anionic and cationic derivatives of cellulose. Using DEAE-cellulose we have succeeded in isolating an apparently homogeneous FSH from swine pituitaries which has high biological activity.

Highly purified FSH (1, 2) having an activity of 8–9 times the Armour Standard (264-151-X) was placed on DEAE-cellulose at neutral pH and low ionic strength. Using gradient elution with 0.5 *M* NaCl-0.1 *M* Na_2HPO_4 it was possible to concentrate the activity several fold. The resulting product was dialyzed and rechromatographed using a lower ionic strength gradient. Only one fraction possessed activity. Subsequently chromatography of this product showed it to act as a homogeneous material.

Preliminary physico-chemical studies have been conducted on the above product. Ultracentrifugal studies have shown it to behave as a single substance with a sedimentation constant (S_{20w}) of 2.49. The diffusion constant (D_{20w}) was found to be 7.43×10^{-7} and the calculated molecular weight was 29,000. Using paper electrophoresis at pH's between 4.0 and 9.5 there was no evidence of heterogeneity.

Amino acid analysis showed that the product had an approximate cystine content of 6–7% which is close to that of insulin. The total content of carbohydrate was 7–8%, about one-half of which was found to be hexosamine. Mannose, galactose and fucose were detected by paper chromatography. The last carbohydrate has not previously been reported as present in pituitary hormones.

Biological assays have been conducted in hypophysectomized and normal rats. It has 35–50 times the FSH activity of the Armour Standard by the augmentation method of Steelman and Pohley (6). A total dose of 0.001–0.0025 mg. will significantly increase the ovarian weight of the immature hypophysectomized female rat. Assays for luteinizing hormone activity in immature hypophysectomized male rats showed that at a total dose of 0.5 mg. the weight of the ventral prostate was not significantly increased. The ventral prostates had no androgenic morphologic change. Tests for TSH, ACTH and STH at the same dosage level were negative.

Details of the preparation and properties will be published at a later date.

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INFLUENCE OF THE EXTIRPATION OF MALE ACCESSORIES ON THE OCCURRENCE OF A "SOFT CALCULUS" IN THE URINARY BLADDER OF THE RAT

The experiments reported below were carried out to determine if the soft amorphous mass first described by Smith (2) in the urinary bladder of the rat was related to the activity of the male sex accessories. This mass, hereafter referred to as "soft calculus," is a solitary, pear shaped structure, the apex of which points towards, and sometimes extends into the urethra (Fig. 1). Its dimensions are 2 to 10 mm. in length, 1 to 4 mm. in width and 3 to 80 mg in weight. Histologically, it is either finely granular or compact, and shows no recognizable cells, although a nucleus can occasionally be identified. Chemically, the calculus is insoluble in water, ether, alcohol and acetone, but soluble in 3% NaOH and 6% HCl, gives a positive reaction with the biuret, xanthoproteic and

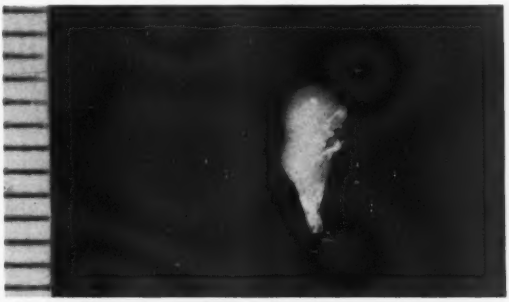

Fig. 1. Photomicrograph of a typical "soft calculus" from the urinary bladder of the male rat (×5). The lower portion of the calculus tapers into the urethral orifice, while the upper, rounded portion has a shape matching the cavity of the bladder.

Millon tests for protein, and with the carbazole test for carbohydrates (1.5%) carried out as indicated by Glegg (1).

Since Smith (2) had mentioned that males infected with *Trichosomoides Crassicauda* had a "preponderance" of calculi, a record of the sex of rats harbouring such a calculus was kept and, in some cases, a search for parasites was carried out. In the 24 female rats studied no calculus was found, while one each was present in 31 out of the 88 males investigated. In a second group of 48 male rats, the bladders of which were serially sectioned and found to be free of parasites, a calculus was frequently present. The soft calculus, therefore, occurred exclusively in males and appeared to be independent of parasites.

The role of the male accessory sex glands in the formation of the calculus was investigated in 36 male rats weighing 220–265 gm. After laparotomy, the presence of a

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calculus in the unopened bladder was ascertained by transillumination with a pencil light, and in half of the animals the seminal vesicles and coagulating glands were extirpated. Two weeks later, the animals were sacrificed (Table 1). A calculus was then found in 8 of the 18 controls, but none in any of the gland extirpated animals, even when one had been recorded before operation. Therefore, either the seminal vesicles or the coagulating glands or both, were essential to calculus formation and maintenance.

A second experiment consisted in extirpating only the seminal vesicles in eight of 16 albino rats weighing between 240 and 320 gm. At autopsy two weeks later (Table 2), a

TABLE 1. INFLUENCE OF THE EXTIRPATION OF SEMINAL VESICLES AND COAGULATING GLANDS ON THE OCCURRENCE OF A "SOFT CALCULUS" IN THE BLADDER OF THE RAT

Gland extirpated	Presence of calculus before operation	Number of animals per group	Number of animals with calculi at autopsy	Average weight of calculi present (mg.)
None	None	13	5	7.1
None	Yes	5	3	12.7
Seminal vesicles + coagulating glands	None	13	0	—
Seminal vesicles + coagulating glands	Yes	5	0	—

calculus could be found in both controls and operated animals, although the calculi found in the latter were very small. Thus, seminal vesicles are not indispensable for the formation of the calculus, but their secretions, when present, may add to its bulk. Presumably, therefore, only the coagulating glands would seem to be indispensable for calculus formation.

A mechanism of calculus formation is suggested by the experiments of Walker (3) who showed that in the rat the secretion of the coagulating glands clots that of the seminal vesicles. Perhaps, the secretions of the seminal vesicles and coagulating glands leak from the prostatic urethra back into the bladder, where their interaction gives rise to a clot—the soft calculus. However, since a small calculus may be found in the absence of seminal vesicles, a calculus might also be composed of the secretion of the coagulating glands alone or in combination with other materials such as (a) prostatic secretion, (b) spermatozoa occasionally seen in the bladder of the rat, and/or (c) precipitated protein of urinary origin known to exist in this species.

TABLE 2. INFLUENCE OF THE EXTIRPATION OF SEMINAL VESICLES ON THE OCCURRENCE OF A "SOFT CALCULUS" IN THE BLADDER OF THE RAT

Gland extirpated	Presence of calculus before operation	Number of animals per group	Number of animals with calculi at autopsy	Average weight of calculi present (mg.)
None	No	6	4	13.8
None	Yes	2	2	15.5
Seminal vesicles	No	4	4	3.0
Seminal vesicles	Yes	4	2	4.0

The occasional disappearance of the calculus in animals with intact glands (second group, Table 2) indicates that the calculus may dissolve in the urine. Under physiological conditions such a loss of material from the calculus may in fact occur continuously. Maintenance of the calculus would then require that the loss be compensated for by further addition of secretory material.

SUMMARY

In the urinary bladder of male but not of female rats, a single "soft calculus" is frequently present. However, no calculus is found after extirpation of both coagulating glands and seminal vesicles. When the seminal vesicles alone are extirpated, a calculus may be found but is much smaller than that found in intact animals. It is concluded that the secretion of the seminal vesicles is helpful and that of the coagulating glands essential for the formation of the soft calculus.

Acknowledgment

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URINARY GLUCOSE AND TUMOR GROWTH IN DIABETIC RATS

Male rats of the Sprague-Dawley strain were subjected to partial pancreatectomy at a weight of approximately 375 gm. Following a period of at least 6 weeks the animals were placed in metabolism cages and force-fed a medium carbohydrate diet (1) by stomach tube each morning and late afternoon (26 cc. per rat per day). Twenty four-hour samples of urine were collected at the same hour each morning and were preserved with thymol. Urine glucose was determined by the method of Benedict (2). Twenty four depancreatized rats excreting from 4 to 6 gm. of glucose per rat per day were matched into two groups, each of which was observed during an additional control period of at least 7 days. At the end of the control period the 12 rats of one group were implanted with

Table 1. Urinary glucose and weights of tumors in force-fed diabetic rats

Experimental condition	No. rats	Total urinary glucose for 3-day period, gm.		Tumor wt., gm. 12-days post implantation
		Last 3 days of control	Last 3 days of expmt.	
Normal	12	no glycosuria	no glycosuria	38.84 ± 2.54
Diabetic, no tumor	12	15.45 ± 0.402	15.37 ± 0.407	
Diabetic, host to tumor	12	14.69 ± 0.449	6.18 ± 0.735	22.72 ± 2.55

Walker carcinoma 256, by injecting saline suspensions of tumor into each groin. Twelve normal force-fed rats of comparable age were also implanted with tumor and kept in metabolism cages. All of the rats were killed after an experimental period of 12 days. The

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data of Table 1 show that the growth of the tumor was suppressed in these severely diabetic rats and that the diabetic rats which were hosts to tumors excreted less glucose towards the end of the experimental period. A full explanation of these data may not be known to us, but it seems apparent that the coexistence of two wasteful, abnormal processes which compete for limited food, will lead to suppression of either one process or of both.

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REFERENCES

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THE COLORIMETRIC ESTIMATION OF THE EPINEPHRINES, DIHYDROXYINDOLES AND THEIR QUINONES WITH AROMATIC ORTHODIAMINES[1]

There is no one accepted chemical technique for measurement of epinephrines in biological material. The more recent assay procedures are based on either a direct or indirect fluorometric measurement. Although fluorescent techniques may be quantitatively applicable for the assay of certain pure compounds, they are not always ideal when dealing with biological material, owing to the presence of nonspecific substances which also fluoresce.

It has been shown that the epinephrines form a condensation product with a mixture of the ethylene diamines which can be measured fluorometrically. This technique has been applied to the assay of blood epinephrines.[2] It has not been possible to employ the same procedure for urine assays for the above mentioned reason of nonspecific fluorescent interference.

It occurred to the writer that if the diamine could be increased in molecular size, a colored product might be formed with the oxidative compounds of the epinephrines and this compound would be measurable on an ordinary laboratory colorimeter. The colored end product should have a specific absorption spectrum, and if the oxidation and condensation reactions took place simultaneously in the same media, difficulties resulting from over oxidation or under oxidation would be overcome.

The condensation products of oxidized epinephrines and the orthodiamines of the aromatic series were studied. It was found that the orthodiamines of benzene and naphthalene formed colored condensation products when added either to epinephrine or norepinephrine in the presence of an oxidizing agent. The catecholamines were oxidized to the quinones of the orthodihydroxyindoles and immediately condensed with the aromatic diamines in acid media at room temperature.

The condensates formed with the phenyleneamines were red, whereas those formed with the naphthylamines were purplish red. The optical densities of the latter were

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[1] Aided by grants from the Lahey Foundation, E. R. Squibb and Sons, and Ciba Pharmaceutical Products.

[2] Weil-Malherbe, H. and Bone, A. D.: The chemical estimation of adrenaline-like substances in blood. *Biochem. J.* **51**: 311–318. 1952.

greater than the former. One absorption maximum suitable for colorimetry of the compound formed in the oxidative reaction of epinephrine and 2,3 naphthalenediamine is approximately 518 $\mu\mu$. The compound has other absorption maximums and these will be reported in a subsequent communication. The condensates may be measured directly, or following extraction and concentration in benzyl alcohol, in a photoelectric colorimeter. The assays were found to be sensitive to a fraction of a gamma of epinephrine. The chemical assays of epinephrine using the 2,3 naphthalenediamine technique measured colorimetrically, were in agreement with bioassays within the error of the latter. (Bioassays on human urines were carried out by measuring the contractile force of the heart in a dog in which a complete sympathetic block was produced by the injection of procaine in the epidural space.)

Certain concentrations of the diamines are necessary for the condensation to go to completion. Linearity and sensitivity of the reaction are, therefore, a function of the concentration of the diamines.

The reaction is most sensitive at a pH of 2.5. Chloride and sulfate ions potentiate, while phthalate inhibits the reaction. Phthalic anhydride and catechol form 1,2- and 2,3-dihydroxyanthraquinones in the presence of sulfuric acid and heat. It is doubtful if this reaction will go to completion with the epinephrines, owing to their heat lability, but it explains the inhibitory effect which may also be produced by a number of organic radicals. The latter will be discussed in detail in a succeeding paper.

Many oxidizing agents may be employed, but the ideal solid substance and the details of its preservation have not been completely worked out. For simplicity, superoxol is recommended at this writing. It should be stored in a refrigerator and a standardization curve[3] run weekly.

The 2,3- position for the amines were the most desirable for the condensation reaction. The condensates have not been isolated and, therefore, the formulae cannot be established. A cyclic bonding of the quinone to the nitrogens in the amines or a phenazine of the indole and the naphthalene equivalents of these compounds are possibilities.

PROCEDURE

2 cc. of 75 mg. % 2,3 diaminonaphthalene dihydrochloride[4] in 0.1 N acetic acid. The solution is allowed to stand at room temperature for a few hours, then overnight in a refrigerator. After bringing to room temperature again, the required amount is centrifuged and only the supernatant fluid used in the assay. The solution is stored in the refrigerator when not in use.

0.1 ml. Saturated KCl.

0.2 ml. Solution of epinephrine, norepinephrine containing 0, 5, 10 or 20 gamma or unknown.

0.1 to 0.2 cc. Superoxol.[3]

Shake, allow to stand at 20 to 25° C and measure at 518 $\mu\mu$ in a spectrophotometer or with a 500 to 520 $\mu\mu$ filter type instrument, subtracting the blank value from the standards. Measurement should start 5 minutes after mixing and continue at 2 minute intervals until maximum color is developed. This usually occurs between 8 and 20 min-

[3] Superoxol activity is periodically checked by running a standardization reaction, varying the superoxol in increments of 0.05 cc.

[4] Since submitting this paper for publication, it was found that different batches of 2,3 diaminonaphthalene dihydrochloride varied in reactivity and this was due to differences in the iron content. Four-hundredths milligram of elemental ferric iron per 100 mg. of the pure crystalline naphthalene salt should be present for optimal results. It may be necessary to vary this quantity of iron slightly with different batches of the naphthalene reagent. The ferric salts of the chloride or nitrate may be used.

utes and is a function of the room temperature. After completion of colorimetric readings, measure pH values which should range between 2.45 and 2.60.

SUMMARY

The orthodiamines of benzene and naphthalene condense with oxidized epinephrine solutions in acid media to form colored compounds. The optical density of the compounds formed with naphthylamines are greater than those formed with the phenyleneamines. A simplified colorimetric microtechnique for the assay of epinephrine and norepinephrine with 2,3 diaminonaphthalene dihydrochloride is described. This method may be employed for the assay of these epinephrines in biological material.

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THE ENDOCRINE SOCIETY

1957 Annual Meeting

The Thirty-ninth Annual Meeting of The Endocrine Society will be held in the Hotel New Yorker, New York, New York, Thursday, Friday, and Saturday, May 30, 31, June 1, 1957.

The Committee on Local Arrangements is Dr. Rulon W. Rawson as Chairman with Drs. Earl T. Engle, Joseph W. Jailer, Warren O. Nelson, and Martin Sonenberg as members of the Committee.

All Scientific Sessions will be held in the Hotel New Yorker. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 31, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the New Yorker, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Eleanor Venning, Royal Victoria Hospital, Montreal, Quebec, Canada, not later than February 1, 1957. *It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting*—in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.
   Line 3. Institution of origin and city in which institution is located.
3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter perfect, since there will be no opportunity for proof reading by authors.

1957 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. Dr. Carl R. Moore was the recipient in 1955 and Dr. Frederick L. Hisaw in 1956.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara M. Szego (Mrs. Roberts); 1954—Dr. I. N. Rosenberg; 1955—Dr. Jack Gross; 1956—Dr. Alfred M. Bongiovanni. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.

2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual and shall be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of the Endocrine Society; the Ciba Award; the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *November 1* each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *November 1, 1956*.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1956.

EIGHTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

THE ENDOCRINE SOCIETY IN COOPERATION WITH THE UNIVERSITY OF TEXAS POSTGRADUATE SCHOOL OF MEDICINE AND THE UNIVERSITY OF TEXAS, M. D. ANDERSON HOSPITAL AND TUMOR INSTITUTE, OCTOBER 22–27, 1956, TEXAS MEDICAL CENTER, HOUSTON 25, TEXAS

PROGRAM OF COURSE

Monday (AM) October 22, 1956

8:00– 9:00 Registration: Place—Clinic Lobby, The University of Texas M. D. Anderson Hospital and Tumor Institute
Introduction to Endocrinology
Chairman—Dr. J. B. Trunnell
9:00– 9:40 General Concepts of Endocrinology, including Neurohumoral Interrelationships—Dr. C. Fortier
9:40–10:20 Chemistry of the Hormones—Dr. A. Albert
10:20–10:35 Intermission
10:35–11:10 Examination of the Patient with Endocrine Disease—Dr. F. Engel
11:10–12:00 Application of Laboratory Procedures to the Diagnosis of Endocrine Disease—Dr. A. Segaloff
12:00–12:30 Principles of Endocrine Therapy—Dr. C. Lloyd
12:30– 2:00 Lunch

Monday (PM)

2:00– 5:30 Panel Discussion with Case Presentations
Hypothalamic-Adeno-Hypophyseal Function and Disease
1. Physiologic Function of the Adenohypophysis
2. Pituitary Tumors—Anatomical and Physiologic Considerations and Relationship to Hypothalamus
3. Pituitary and Hypothalamic Hyperfunction Syndromes—Acromegaly, Gigantism, Precocious Puberty, etc.
4. Pituitary and Hypothalamic Hypofunction Syndromes—Chromophobe Adenoma, Craniopharyngioma, Sheehan's Syndrome, Diabetes Insipidus, etc.

Drs. A. Albert (Moderator), F. Engel, C. Fortier, C. Lloyd, and O. Pearson
7:00 P.M. Get-acquainted Cocktail Party—Doctors' Club, Texas Medical Center Library Building. (Sponsored by E. R. Squibb & Sons, Division of Olin Mathieson Chemical Corporation)

Tuesday (AM) October 23, 1956

9:00–12:30 Panel Discussion with Case Presentation
Spontaneous and Induced Disorders of the Adrenal Cortex
1. Physiological Functions of the Adrenal Cortex
2. Hyperfunction—Cushing's Syndrome
3. Hypofunction Syndromes
   a. Addison's Disease
   b. Iatrogenic Adrenal Insufficiency induced by Steroid Therapy—Diagnosis and Management
   c. Present Status and Management of Patients Adrenalectomized (o:

Hypophysectomized) for Cancer, Hypertension, Diabetes or Cushing's Syndrome

Drs. F. Engel (Moderator), J. Conn, J. Greene, O. Pearson and A. Segaloff

12:30– 2:00 Lunch

Tuesday (PM)

2:00– 5:30 Panel Discussion with Case Presentations

Disorders of Steroid Hormone Synthesis and Metabolism

1. Normal and Abnormal Biogenesis of Steroid Hormones and the Laboratory Evaluation thereof
2. Virilizing Syndromes due to Disorders of Steroid Synthesis
   a. Adrenogenital Syndrome
   b. Virilization due to Abnormal Ovarian Steroid Synthesis—Arrhenoblastoma, Hilar Cell Tumor, Adrenal Rest Tumor
   c. Differentiation from Other Types of Hirsutism
3. Syndromes due to Abnormalities in Testicular Steroid Secretion
   a. Interstitial Cell Tumor
   b. Adrenal Rest Tumor

Drs. A. Segaloff (Moderator), R. Greenblatt, W. Nelson, J. Van Wyk and L. Wilkins

Wednesday (AM) October 24, 1956

9:00–12:30 Panel Discussion with Case Presentations

Disorders of the Thyroid Gland

1. Anatomical and Physiologic Considerations
2. Laboratory Evaluation of Thyroid Function
3. Evaluation of the Patient with Goitre
   a. Colloid Goitre
   b. Adenoma—Single and Multiple, toxic and non-toxic
   c. Cancer of the Thyroid
   d. Thyroiditis
4. Hyperthyroidism with Particular Reference to Treatment and Complications
5. Hypothyroidism
   a. Adult Myxedema
   b. Congenital Hypothyroidism

Drs. R. Rawson (Moderator), A. Albert, J. Trunnell, H. Turner and J. Van Wyk

12:30–2:00 Lunch

Wednesday (PM)

2:00– 5:30 Panel Discussion with Case Presentations

Problems of Growth and Development in Children

1. The Endocrine and Non-endocrine Factors Concerned in Growth and Development
2. Abnormalities in Growth and Development
3. Constitutional Variations in the Pattern of Adolescent Development
4. The Types and Diagnosis of Sexual Precocity
5. Ambisexual Precocity (Hermaphroditism)

Drs. L. Wilkins (Moderator), G. Clayton, T. Panos, W. Sutow and J. Van Wyk

Thursday (AM) October 25, 1956

BONES, STONES, AND CALCIUM METABOLISM

Chairman: DR. D. SELDIN

9:00– 9:50 Calcium, Phosphorus and Bone Metabolism and Physiology of Parathyroids—Dr. R. Talmage
9:50–10:20 Hypoparathyroidism—Dr. J. Howard
10:20–10:35 Intermission
10:35–11:20 Hyperparathyroidism—Dr. F. Engel
11:20–12:00 Metabolic Bone Disease—Dr. J. Howard
12:00–12:30 Questions and Answers
12:30– 2:00 Lunch

Thursday (PM)

THE ENDOCRINE GLANDS AND ELECTROLYTE AND WATER BALANCE

Chairman: DR. J. GREENE

2:00– 2:40 Sodium, Potassium and Water Homeostasis—Dr. D. Seldin
2:40– 3:20 The Adrenal Cortex and Electrolyte Balance—Aldosteronism—Dr. J. Conn
3:20– 3:50 Vasopressin and Water Balance—Diabetes Insipidus—Dr. O. Pearson
3:50– 4:00 Intermission

THE ENDOCRINE GLANDS AND INTERMEDIARY METABOLISM

4:00– 4:30 Endocrine Factors in Diabetes Mellitus—Dr. J. Conn
4:30– 5:00 Hypoglycemia—Dr. J. Howard
5:00– 5:30 Questions and Answers

Friday (AM) October 26, 1956

9:00–12:30 Panel Discussion with Case Presentations
Problems of Gonadal Dysfunction (Female)
1. Physiology of Ovarian Function
2. Laboratory Evaluation of Ovarian Function
3. Ovarian Hypofunction Syndromes, including the Menopause
4. Ovarian Dysfunction Syndromes—Menstrual Disorders—Stein-Leventhal Syndrome
5. Use and Abuse of Sex Hormone Therapy in the Female

Drs. W. Allen (Moderator), R. Greenblatt, C. Lloyd, A. Segaloff and F. Simmons

12:30– 2:00 Lunch

Friday (PM)

2:00– 4:00 Panel Discussion with Case Presentations
Problems of Gonadal Dysfunction (Male)
1. Development and Physiology of the Testis
2. Laboratory Evaluation of Testicular Function
3. Male Hypogonadism
   a. Primary vs. Secondary
   b. Klinefelter's Syndrome
   c. Cryptorchism

Drs. W. Nelson (Moderator), A. Albert, J. Howard, F. Simmons and H. Turner

4:00– 4:15 Intermission

4:15– 5:30 Panel Discussion—Evaluation of Infertility and Failure of Reproductive Efficiency

1. Male Infertility
2. Female Infertility
3. Habitual Abortion, Toxemia of Pregnancy

Drs. F. Simmons (Moderator), W. Allen, C. Lloyd and W. Nelson

8 P.M. Banquet of the Course, including students and faculty. Doctors' Club, Texas Medical Center Library Building. Cost: $4.25. Tickets will be sold at time of registration.

Speaker: Dr. Warren Nelson—"Endocrinology and World Population"

Saturday (AM) October 27, 1956

9:00–10:30 Demonstration of Laboratory Procedures and Special Techniques and Visit to The University of Texas M. D. Anderson Hospital and Tumor Institute

Drs. R. Greenblatt, J. Trunnell and G. Clayton

10:30–10:45 Intermission

10:45–12:30 Questions and Answers—The Faculty

FACULTY

A. Albert, M. D., Professor of Physiology, Mayo Foundation; Head, Endocrinology Laboratory, Mayo Clinic.

Willard M. Allen, M.D., Professor of Obstetrics and Gynecology, Washington University School of Medicine.

George W. Clayton, M.D., Instructor of Pediatrics, Baylor Medical School.

Jerome W. Conn, M.D., Professor of Medicine, Director, Division of Endocrinology and Metabolism, University of Michigan.

Frank L. Engel, M.D., Associate Professor of Medicine, Assistant Professor of Physiology, Duke University School of Medicine; Director, Division of Endocrinology, Duke Hospital

Claude Fortier, M.D., Ph.D., Director, Laboratories of Neuroendocrinology, Blue Bird Children's Clinic for Neurological Disorders; Associate Professor of Physiology, Baylor University College of Medicine.

James A. Greene, M.D., Professor and Chairman, Department of Medicine, Baylor University College of Medicine, and Clinical Professor of Medicine, University of Texas Postgraduate School of Medicine.

Robert A. Greenblatt, M.D., Professor of Endocrinology, Medical College of Georgia.

John Eager Howard, M.D., Associate Professor of Medicine, Johns Hopkins University, School of Medicine.

Charles W. Lloyd, M.D., Associate Professor of Obstetrics and Associate Professor of Medicine, Division of Endocrinology, State University of New York, Upstate Medical Center.

Warren O. Nelson, M.D., Medical Director, Population Council, Rockefeller Institute; Professor of Anatomy, University of Iowa (on leave of absence).

Theodore C. Panos, M.D., Professor of Pediatrics, The University of Texas Medical Branch.

Olof H. Pearson, M.D., Associate Professor of Medicine, Cornell University Medical College, Associate Physician, Memorial Hospital.

Rulon W. Rawson, M.D., Executive Officer, Department of Medicine, Memorial Center; Chief, Division of Clinical Investigation, Sloan-Kettering Institute, Professor of Medicine, Cornell University Medical School.

Donald W. Seldin, M.D., Professor and Chairman, Department of Internal Medicine, The University of Texas Southwestern Medical School.

Fred A. Simmons, M.D., Instructor in Gynecology, Harvard Medical School, Associate Visiting Surgeon, Massachusetts General Hospital.

Albert Segaloff, M.D., Director of Endocrine Research, Alton Ochsner Medical Foundation.

Wataru W. Sutow, M.D., Associate Pediatrician, The University of Texas, M. D. Anderson Hospital and Tumor Institute; Associate Professor of Pediatrics, The University of Texas Postgraduate School of Medicine; Clinical Assistant Professor of Pediatrics, Baylor University School of Medicine.

Roy V. Talmage, Ph.D., Professor of Biology, Rice Institute.

J. B. Trunnell, M.D., Head, Section of Experimental Medicine, The University of Texas, M. D. Anderson Hospital and Tumor Institute; Associate Professor of Medicine, The University of Texas Postgraduate School of Medicine.

Henry H. Turner, M.D., Clinical Professor of Medicine, University of Oklahoma School of Medicine; Chief, Endocrine Clinic, University Hospitals.

Judson J. Van Wyk, M.D., Assistant Professor of Pediatrics, University of North Carolina School of Medicine; Assistant Attending Pediatrician, North Carolina Memorial Hospital.

Lawson Wilkins, M.D., Associate Professor of Pediatrics, Johns Hopkins University School of Medicine.

UROLOGY AWARD

The American Urological Association offers an annual award of $1000 (first prize of $500, second prize $300 and third prize $200) for essays on the result of some clinical or laboratory research in Urology. Competition shall be limited to urologists who have been graduated not more than ten years, and to hospital internes and residents doing research work in Urology.

The first prize essay will appear on the program of the forthcoming meeting of the American Urological Association, to be held at the Hotel William Penn, Pittsburgh, Pennsylvania, May 6–9, 1957.

For full particulars write the Executive Secretary, William P. Didusch, 1120 North Charles Street, Baltimore, Maryland. Essays must be in his hands before December 1, 1956.

COLLEGE OF AMERICAN PATHOLOGISTS

The 1956 International Cancer Cytology Congress will be held at the Drake Hotel in Chicago, Illinois October 8 to 13, 1956.

This Congress, the first of its kind, will be sponsored by the American Society of Clinical Pathologists, the College of American Pathologists, the Intersociety Cytology Council and the International Union Against Cancer. The first three organizations comprise a combined membership of approximately three thousand pathologists and other physicians. It is expected that a number of guests from Europe, South America and Latin American countries will be present at this Congress as representatives of the International Union Against Cancer.

The general theme of the Congress will be Exfoliative Cytology and the program to be presented during this period will stress but not be devoted exclusively to the subject of Exfoliative Cytology in all of its various aspects and its relationship to various specialties of medicine.

During this period three of the sponsoring organizations will hold their annual meeting, as follows:

The College of American Pathologists the evening of Monday, October 8

The Intersociety Cytology Council the evening of Wednesday, October 10

The American Society of Clinical Pathologists the evening of Thursday, October 11

A joint banquet of all organizations will be held on the evening of Tuesday, October 9

AMERICAN PSYCHOSOMATIC SOCIETY
FOURTEENTH ANNUAL MEETING

The American Psychosomatic Society will hold its Fourteenth Annual Meeting at Chalfonte-Haddon Hall in Atlantic City on Saturday and Sunday, May 4 and 5, 1957.

The Program Committee would like to receive titles and abstracts of papers for consideration for the program no later than December 1, 1956. The time allotted for presentation of each paper will be twenty minutes.

Abstracts, in sextuplicate, should be submitted for the Program Committee's consideration to the Chairman, at 551 Madison Avenue, New York 22, New York.

I. ARTHUR MIRSKY, M.D., *Chairman, Program Committee*